Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Endothelial Lipase Is a Critical Determinant of High-Density Lipoprotein–Stimulated Sphingosine 1-Phosphate –Dependent Signaling in Vascular Endothelium

Satoru Tatematsu, Sanjeev A. Francis, Pradeep Natarajan, Daniel J. Rader, Alan Saghatelian, Jonathan D. Brown, Thomas Michel and Jorge Plutzky

Arterioscler Thromb Vasc Biol. 2013;33:1788-1794; originally published online May 30, 2013;
doi: 10.1161/ATVBAHA.113.301300
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

Endothelial Lipase Is a Critical Determinant of High-Density Lipoprotein–Stimulated Sphingosine 1-Phosphate–Dependent Signaling in Vascular Endothelium

Satoru Tatematsu,* Sanjeev A. Francis,* Pradeep Natarajan, Daniel J. Rader, Alan Saghatelian, Jonathan D. Brown, Thomas Michel, Jorge Plutzky

Objective—In addition to an extensively characterized role of high-density lipoprotein (HDL) in reverse cholesterol transport, bioactive lipids bound to HDL can also exert diverse vascular effects. Despite this, integration of HDL action in the vasculature with pathways that metabolize HDL and release bioactive lipids has been much less explored. The effects of HDL on endothelial cells are mediated in part by HDL-associated sphingosine 1-phosphate (S1P), which binds to $S1P_1$ receptors and promotes activation of endothelial NO synthase (eNOS) and the kinase Akt. In these studies, we characterized the role of endothelial lipase (EL) in the control of endothelial signaling and biology, including those mediated by HDL-associated S1P.

Approach and Results—HDL-induced angiogenesis in aortic rings from EL-deficient ($EL^{-/-}$) mice was markedly decreased compared with wild-type controls. In cultured endothelial cells, small interfering RNA–mediated knockdown of EL abrogated HDL-promoted endothelial cell migration and tube formation. Small interfering RNA–mediated EL knockdown also attenuated HDL-induced phosphorylation of $eNOS^{1179}$ and Akt^{473}. S1P stimulation restored HDL-induced endothelial migration and Akt/eNOS phosphorylation that had been blocked by small interfering RNA–mediated EL knockdown. HDL-induced endothelial cell migration and Akt/eNOS phosphorylation were completely inhibited by the $S1P_1$ antagonist W146 but not by the $S1P_3$ antagonist CAY10444.

Conclusions—EL is a critical determinant of the effects of HDL on S1P-mediated vascular responses and acts on HDL to promote activation of $S1P_1$, leading to Akt/eNOS phosphorylation and subsequent endothelial migration and angiogenesis. The role of EL in HDL-associated S1P effects provides new insights into EL action, the responses seen through EL and HDL interaction, and S1P signaling. **(*Arterioscler Thromb Vasc Biol*. 2013;33:1788-1794.)**

Key Words: angiogenesis ■ endothelial cells ■ endothelial lipase, mouse ■ HDL cholesterol ■ sphingosine 1-phosphate

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are inversely associated with the risk of cardiovascular diseases.[1] The antiatherogenic effect of HDL is attributed at least in part to reverse cholesterol transport.[2] In addition, multiple lines of evidence indicate that other salutary effects of HDL on endothelial cells (ECs) include antioxidative, anti-inflammatory, antithrombotic, and proangiogenic effects.[3–5] HDL is the most quantitatively important plasma carrier of sphingosine-1-phosphate (S1P), which is a bioactive lipid molecule. HDL-associated S1P has been demonstrated to be involved in a broad range of vascular responses[6–9] modulated by a family of ≥5 different S1P receptor subtypes identified in mammals; ECs express most prominently the $S1P_1$ and $S1P_3$ receptor isoforms.[9,10] It was recently reported that HDL-induced Akt activation and endothelial responses are mediated by $S1P_1$ in human umbilical vein ECs.[11] In contrast, other studies suggest that HDL-induced Akt activation is mediated by $S1P_3$ receptors.[12] The identity of the S1P receptor subtype that modulates HDL responses in the endothelium remains unsettled, and the mechanisms whereby S1P is released from HDL is unclear. The studies presented here explore the roles of endothelial lipase (EL) and the identity of the S1P receptor subtype in determining HDL-dependent responses in the vascular wall.

EL is a member of the triglyceride lipase family, which includes lipoprotein lipase and hepatic lipase. EL is principally a phospholipase, with nominal triglyceride lipase activity.[13,14] EL is synthesized principally by ECs[15] and hydrolyzes

Received on: June 1, 2012; final version accepted on: May 13, 2013.

From the Division of Cardiovascular Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA (S.T., S.A.F., P.N., J.D.B., T.M., J.P.); Division of Translational Medicine and Human Genetics, University of Pennsylvania, Philadelphia, PA (D.J.R.); and Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA (A.S.).

*These authors contributed equally.

This manuscript was sent to Qingbo Xu, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.113.301300/-/DC1.

Correspondence to Jorge Plutzky, Brigham and Women's Hospital, 77 Ave Louis Pasteur, NRB 742, Boston, MA 02115 (e-mail jplutzky@rics.bwh.harvard.edu); or Thomas Michel, Brigham and Women's Hospital, 20 Shattuck St, Thorn 1210A, Boston, MA 02115 (e-mail thomas_michel@harvard.edu).

HDL much more efficiently than other lipoproteins.[16] Plasma HDL levels are increased in EL knockout ($EL^{-/-}$) mice and are decreased in EL transgenic mice.[17] Furthermore, the N396S variant in the human EL gene (*LIPG*) shows reduced lipase activity and is associated with elevated HDL-C levels.[18] Another EL variant, G26S EL shows reduced plasma levels of EL protein.[19] Yet the role of EL in atherosclerosis remains controversial. Inactivation of EL increases the plasma HDL-C levels and inhibits atherosclerosis in $ApoE^{-/-}$ mice.[20] In contrast, another study reported that a deficiency of EL expression does not affect atherosclerosis in either $ApoE^{-/-}$ mice or $LDLR^{-/-}$ mice, although plasma HDL-C levels are elevated.[21] Broedl et al[22] have shown that EL overexpression results in reduced very-low-density lipoprotein/low-density lipoprotein cholesterol and phospholipid levels. In addition, we have previously reported that HDL hydrolysis by EL activates peroxisome proliferator activated receptor-α and represses vascular cell adhesion molecule 1 expression in ECs, which may contribute to the anti-inflammatory effects of HDL.[23] These multiple lines of evidence suggest EL action might limit atherosclerosis. Several genetic association studies have shown conflicting results about the association between common genetic variants in *LIPG* gene and the risk of cardiovascular diseases. Vergeer et al[24] reported that the T111I variant in the *LIPG* gene is associated with higher HDL-C levels but is not related to cardiovascular disease risk. Moreover, recent Mendelian randomization analysis studies in 20 913 myocardial infarction cases compared with 95 407 controls found that a single nucleotide polymorphism in the EL gene significantly increased HDL-C levels but conferred no protection against myocardial infarction.[25] These findings raise questions about the connection between plasma HDL-C levels, per se, and protection against atherosclerosis and the connection between the enzymatic function of EL and the vascular effects of HDL.

Sphingosine 1-phosphate (S1P) is a bioactive lipid that binds to a family of G protein–coupled receptors that modulate signaling responses in multiple cells and tissues.[26] $S1P_1$ receptors in the vascular endothelium are reversibly targeted to plasmalemmal caveolae and promote the activation of kinase Akt and of the endothelial isoform of NO synthase (eNOS), leading to vasorelaxation.[27] The EC_{50} for S1P-promoted eNOS phosphorylation is ≥1 order of magnitude lower than the plasma concentration of S1P, reflecting the fact that plasma S1P is mostly bound to plasma proteins. HDL particles represent the predominant S1P-carrier in plasma, with recent studies revealing apolipoprotein M in HDL as a specific S1P-binding protein.[28,29]

The roles of EL in modulating HDL-dependent signaling responses via S1P have not been well characterized. The current studies use experiments in ex vivo vascular preparations as well as in cultured ECs to test the hypothesis that HDL hydrolysis by EL induces angiogenesis and stimulates endothelial signaling responses via $S1P_1$ receptors in the vascular endothelium.

Materials and Methods

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

Results

EL Is Involved in HDL-Induced Endothelial Proliferation, Tube Formation, and Angiogenesis

Given EL as the predominant lipase expressed by ECs, we first studied the effects of the general lipase inhibitor tetrahydrolipstatin on HDL-induced EC migration. Addition of HDL (100 μg/mL) approximately doubled EC migration as compared with vehicle stimulation, an effect blocked by tetrahydrolipstatin-mediated lipase inhibition (Figure 1A and 1B). To more directly investigate the role of EL in HDL-induced cell migration, bovine aortic ECs were transfected with a duplex small interfering RNA (siRNA) construct targeting EL. Bovine aortic ECs transfection with EL siRNA reduced EL mRNA by 90% and EL protein abundance by 50% reduction (Figure IA in the online-only Data Supplement), but had no effect on eNOS, Akt, AMP-activated protein kinase, ERK1/2, p38MAPK, or PTEN protein levels (Figure IB in the online-only Data Supplement). siRNA-mediated EL knockdown suppressed HDL-promoted EC migration (Figure 1C and 1D); control siRNA had no effect on the HDL-induced migration response. Viability assays revealed that HDL increased EC viability but not after siRNA-mediated EL knockdown (Figure 1E). Similarly, HDL stimulation increased EC proliferation (Figure 1F) but not after EL siRNA exposure.

HDL stimulation of ECs promoted endothelial tube formation, which was also inhibited by siRNA-mediated EL knockdown, as seen on microscopy (Figure 2A) and after quantification of the average total tube length per field of view (Figure 2B).

Next, we used ex vivo angiogenesis in aortic rings model to evaluate the role of EL in HDL-induced angiogenesis. HDL markedly stimulated angiogenesis in aortic rings isolated from wild-type (WT) mice but not $EL^{-/-}$ knockout mice (Figure 3A), as quantified using the total area of new vessel formation (Figure 3B).

siRNA-Mediated EL Knockdown Inhibits HDL-Induced Phosphorylation Responses

To elucidate the signaling pathways responsible for these HDL-mediated EL-dependent migration and angiogenesis responses, we examined the phosphorylation pattern of several key endothelial signaling proteins in response to HDL in the presence or absence of EL RNA interference. As shown in Figure 4, HDL treatment promoted the time- and dose-dependent phosphorylation of eNOS Ser^{1179} and of kinase Akt, yielding a 2.0-fold (n=3; $P<0.01$) increase in eNOS phosphorylation and a 3.7-fold (n=3; $P<0.01$) increase in Akt phosphorylation. siRNA-mediated EL knockdown attenuated HDL-induced eNOS and Akt phosphorylation, as assessed both in dose–response (Figure 4A) and time course (Figure 4B) experiments.

HDL-Stimulated EL-Dependent Signaling Responses Are Mediated by $S1P/S1P_1$

S1P signals through distinct S1P receptor subtypes. We used S1P receptor subtype-selective antagonists to assess the role of HDL-bound S1P in HDL-induced EC migration (Figure 5) and Akt/eNOS phosphorylation (Figure 6A) responses mediated by EL. The $S1P_1$ antagonist W146 blocked HDL-induced EC migration and Akt/eNOS phosphorylation. In contrast, the

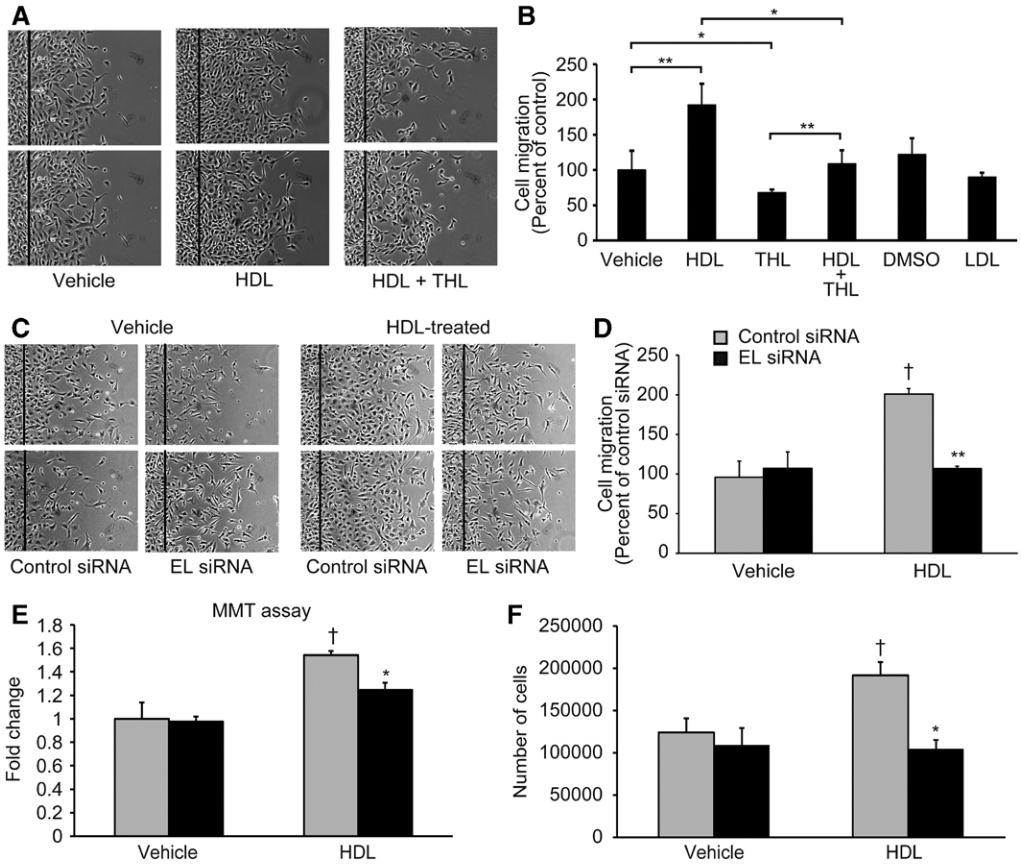


Figure 1. Inhibition of lipase activity attenuates high-density lipoprotein (HDL)–induced endothelial cell (EC) migration and proliferation. **A**, EC migration assay after treatment with HDL (100 μg/mL) in the presence or absence of the lipase inhibitor tetrahydrolipstatin (THL; 10 μmol/L). Dimethyl sulfoxide (DMSO; 0.1%) and low-density lipoprotein (LDL; 100 μg/mL) were used as controls. **B**, Quantification of EC migration was determined by the number of the cells that populated the area (n=4 per condition). **P*<0.05 vehicle vs THL and HDL vs THL+HDL, ***P*<0.01 vehicle vs THL and HDL vs THL+HDL. **C**, EC migration assays were analyzed in bovine aortic ECs transfected with control or endothelial lipase (EL) small interfering RNA (siRNA). **D**, Quantification of EC migration as determined by the number of cells that populated the area (n=4 per condition). ***P*<0.01 vs control siRNA with the same treatment, †*P*<0.05 vs control siRNA with vehicle. **E**, MTT viability assay after transfection with control siRNA or EL siRNA and treatment with HDL (75 μg/mL; n=4 per condition) are shown as fold change vs cells exposed to control siRNA/vehicle. **P*<0.05, †*P*<0.05 vs control siRNA/vehicle. **F**, Effect of EL deficiency on the proliferative response to HDL by counting the number of cells after a 24-hour treatment (n=3 per condition). **P*<0.05 vs control siRNA with the same treatment, †*P*<0.05 vs control siRNA with vehicle.

$S1P_3$ antagonist CAY10444 did not alter either HDL-induced EC migration or Akt/eNOS phosphorylation. Direct stimulation of cells with S1P itself reversed the inhibition seen with EL siRNA on both HDL-induced EC migration (Figure 5) and Akt/eNOS phosphorylation (Figure 6). Given these findings that EL can modulate HDL-induced endothelial responses via S1P, we sought to investigate differences in S1P levels in plasma from WT and EL-deficient mice. After developing and validating a liquid chromatography, tandem mass spectroscopy method in our laboratory for detecting S1P levels in murine plasma, we documented S1P concentrations in murine plasma in the high nanomolar range (Figure II in the online-only Data Supplement). S1P plasma levels did not differ between WT (n=4) and EL-deficient male mice (n=6; Figure 6B).

Discussion

We have used siRNA and pharmacological approaches in cultured ECs, as well as analyses using knockout mouse models, to study the roles of EL and S1P in HDL-induced angiogenesis and signaling responses. We found that EL loss-of-function, through either the lipase inhibitor tetrahydrolipstatin or through siRNA-mediated EL knockdown, markedly attenuated HDL-induced EC migration (Figure 1A–1D), proliferation (Figure 1E and 1F), and tube formation (Figure 2). HDL-induced angiogenesis was also inhibited in ex vivo studies of aortic rings from EL-deficient mice (Figure 3). HDL-promoted Akt and eNOS phosphorylation was markedly inhibited after siRNA-mediated EL knockdown (Figure 4). The effects of EL/HDL on EC migration (Figure 5) as well as Akt and eNOS phosphorylation (Figure 6) were blocked by an antagonist to $S1P_1$ but not $S1P_3$, whereas S1P stimulation reversed the inhibition of these endothelial responses seen with EL siRNA exposure. Taken together, these experiments, using lipase inhibitors, siRNA approaches, and a genetic mouse model, strongly implicate EL as a key determinant of HDL-mediated physiological responses in the endothelium.

HDL elicits a broad range of physiological and signaling responses in the endothelium.[30,31] Importantly, HDL may induce substantively different responses in distinct vascular beds and experimental models. For example, HDL promotes tube formation through Ras/ERK1/2 signaling pathway in human coronary artery ECs,[3] whereas Akt-dependent

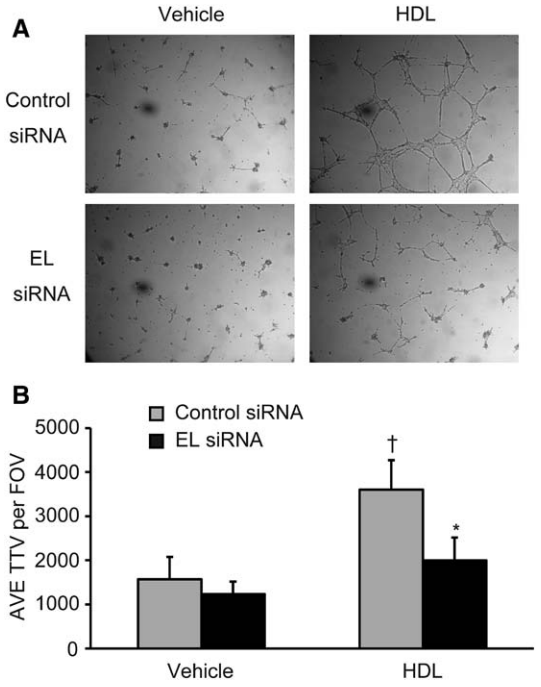


Figure 2. Small interfering RNA (siRNA)–mediated knockdown of endothelial lipase (EL) attenuates high-density lipoprotein (HDL)–induced tube formation. **A**, Effect of EL knockdown on tube formation induced by HDL (75 μg/mL). **B**, Quantification of tube formation as determined by average total tube length (TTL) per field of view (FOV; n=5 per condition). **P*<0.05 vs control siRNA with the same treatment, †*P*<0.05 vs control siRNA with vehicle. Data represent mean±SEM.

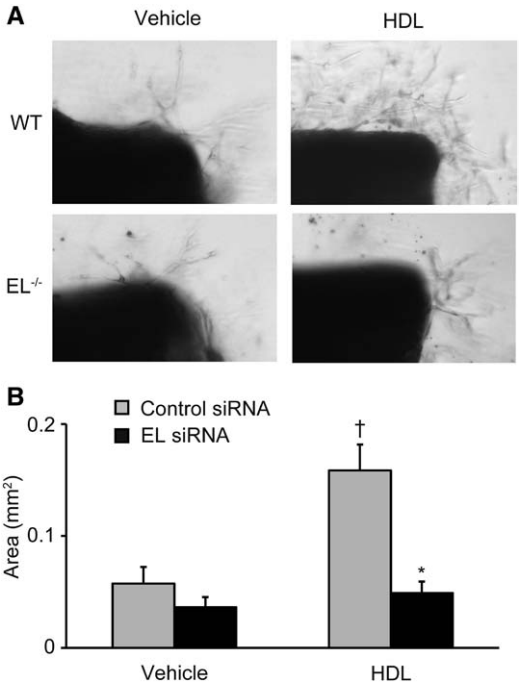


Figure 3. High-density lipoprotein (HDL)–induced angiogenesis is impaired in aortic preparations from the EL$^{-/-}$ knockout mouse. **A**, Angiogenesis from the aortic rings. **B**, Vessel growth area quantification (n=11–12 per condition). **P*<0.05 vs control small interfering RNA (siRNA) with the same condition, †*P*<0.05 vs wild type (WT) with vehicle. Data represent mean±SEM.

pathways seem to modulate HDL responses in human umbilical vein ECs.[32] Other studies implicate AMP-activated protein kinase in HDL responses, including eNOS phosphorylation.[33] The role of EL in mediating these and other HDL effects has not been well explored. Our findings suggest that differential expression and activity of EL may be an under-recognized variable influencing HDL responses seen in various experimental models and perhaps in vivo as well.

The roles of EL-mediated HDL hydrolysis in atherosclerosis remain incompletely understood, with unresolved controversies in the field. Hara et al[34] reported that HDL from EL knockout mice retained the anti-inflammatory features seen in HDL isolated from WT mice. Moreover, Riederer et al[35] reported that lysophosphatidylcholine generated by the action of EL on HDL induced the expression of interleukin-8, a proinflammatory and proadhesive chemokine.[24] These results lend support to the view that hydrolysis of HDL by EL has proinflammatory properties. In contrast, we previously reported that EL overexpression decreased tumor necrosis factor-α–induced vascular cell adhesion molecule 1 expression and promoter activity through peroxisome proliferator activated receptor-α activation in ECs, thus limiting vascular inflammation.[23] Interestingly, individuals with EL–loss-of-function variants have higher HDL but no apparent protection against atherosclerosis or its complications.[25] In the present study, we have shown that HDL exposure to EL induces angiogenesis and promotes Akt/eNOS activation. Angiogenic therapies have been explored for treating cardiovascular diseases, yet clearly a complex, dynamic balance of pro- and antiangiogenic pathways exist and may vary in different physiological and pathological settings.[36] Our data suggest HDL and changes in EL expression and function as potential contributors to these patterns. Further elucidation about mechanisms through which HDL and HDL interaction with EL exert biological effects may provide insight into divergent data sets about the impact of HDL, HDL-modulating therapy, and genetic EL variants on atherosclerosis.

The data provided here implicate S1P as a possible mediator of responses induced by HDL through EL action. The $S1P_1$ receptor antagonist W146 blocked HDL-induced EC migration as well as Akt/eNOS phosphorylation, whereas the $S1P_3$ antagonist CAY10444 did not affect either HDL-induced EC migration or Akt/eNOS phosphorylation. Decreased EL levels, as achieved with siRNA, inhibited HDL-induced EC migration and Akt/eNOS phosphorylation, responses that were restored by adding back S1P (Figures 5 and 6). Our results support the hypothesis of S1P as a key HDL constituent generated by EL that can induce angiogenesis and elicit Akt/eNOS signaling responses. S1P and its receptors have been implicated in HDL-stimulated Akt/eNOS phosphorylation.[11,32,35] We previously reported that free S1P activates eNOS through phosphoinositol 3-kinase/Akt signaling pathways,[27,37] a finding consistent with the current studies showing that HDL-associated S1P elicits similar effects. It is quite likely that other bioactive lipids in addition to S1P are liberated after hydrolysis of HDL by EL. Indeed, the precise mechanism(s) through which EL promotes HDL-dependent S1P release and signaling remains to be determined. EL reportedly catalyzes the conversion of large HDL2 particles to small HDL3 particles, which are more

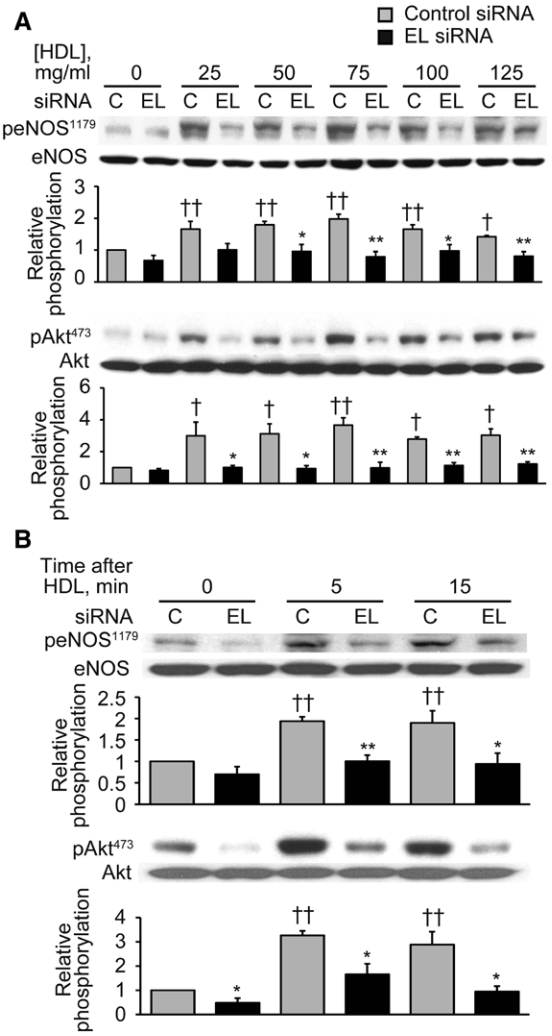


Figure 4. Small interfering RNA (siRNA)–mediated endothelial lipase (EL) knockdown inhibits high-density lipoprotein (HDL)–induced phosphorylation of endothelial NO synthase (eNOS) and Akt. **A**, Immunoblots were analyzed in either vehicle- or HDL-treated bovine aortic endothelial cells (BAECs) transfected with control or EL siRNA. Cell lysates were harvested at 5 minutes after addition of different amounts of HDL as indicated and analyzed in immunoblots probed with specific antibodies against Ser^{1179}-phospho-eNOS ($peNOS^{1179}$), Ser^{473}-phospho-Akt ($pAkt^{473}$), and total eNOS and Akt. The experiment shown is a representative of 3 independent experiments. *P<0.05, **P<0.01 vs control siRNA with the same dose of HDL, †P<0.05, ††P<0.01 vs control siRNA without HDL treatment. **B**, Immunoblots were analyzed in BAECs transfected with control or EL siRNA. Cell lysates were harvested at the indicated times after addition of HDL (75 μg/mL) and analyzed in immunoblots probed with specific antibodies against Ser^{1179}-phospho-eNOS ($peNOS^{1179}$), Ser^{473}-phospho-Akt ($pAkt^{473}$), and total eNOS and Akt. The experiment shown is a representative of 3 independent experiments. *P<0.05, **P<0.01 vs control siRNA with the same dose of HDL, ††P<0.01 vs control siRNA without HDL treatment (0 minutes). Data represent mean±SEM.

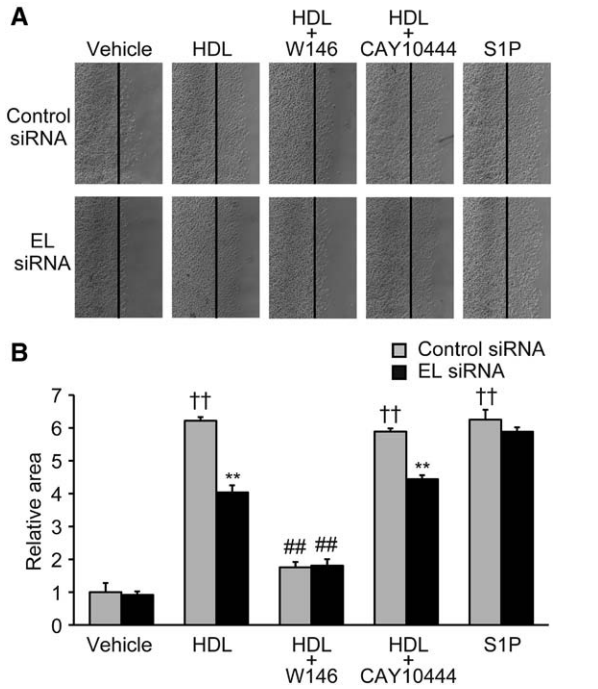


Figure 5. Sphingosine 1-phosphate (S1P)/$S1P_1$ is involved in high-density lipoprotein (HDL)–induced endothelial cell (EC) migration mediated by endothelial lipase. **A**, EC migration assay after treatment with vehicle, HDL (75 μg/mL), HDL (75 μg/mL) + W146 (10 μmol/L), HDL (75 μg/mL) + CAY10444 (10 μmol/L), or S1P (100 nmol/L) in bovine aortic endothelial cells transfected with control or endothelial lipase (EL) small interfering RNA (siRNA). **B**, Quantification of EC migration as determined by the area of migrated cells (n=4 per condition). **P<0.01 vs control siRNA with the same treatment, ††P<0.01 vs control siRNA with vehicle, ##P<0.01 vs HDL with the same siRNA. Data represent mean±SEM.

highly enriched in S1P relative to large HDL2 particles.[38–40] In addition, EL may also augment S1P effects by reducing S1P catabolism, inhibiting S1P lyase, or facilitating S1P delivery.

Based on our findings, it will be of particular interest to consider how apolipoprotein M, as a recently identified S1P-binding partner in HDL,[28,29] might be involved in the EL-mediated effects reported here. One recent studied analyzed S1P plasma levels in relationship to apolipoprotein M in different monogenic disorders of HDL metabolism.[41] In that work, considerable variability in S1P levels was seen among the 10 Dutch patients with LIPG (EL) gene variations, which the authors speculated could have resulted from various issues. Our inability to detect changes in S1P levels in WT compared with EL-deficient mouse plasma may have been influenced by multiple factors. Total plasma S1P reflects heterogenous protein-bound S1P pools. The levels of S1P actually bound to isolated murine HDL might be a more relevant measure, but has not been routinely performed in the field and remains technically challenging (data not shown). The total plasma S1P concentration in mice does far exceed the EC50 for S1P-promoted Akt phosphorylation or eNOS activation (2–10 nmol/L),[37] arguing for the biological plausibility of this mechanism. The possibility that EL might be involved in S1P release from HDL, either directly or indirectly, has been raised in various models[38] but, to our knowledge, the results presented here are the first that directly support this prospect. Of note, ABCA1, ABCG1, and SR-B1, important transporter proteins in HDL pathways, have all been implicated in S1P handling.[39]

We also provide evidence that S1P released from HDL by EL activates $S1P_1$ receptors that promote Akt/eNOS activation. Although other model systems have implicated both $S1P_3$ and $S1P_1$ receptors in these responses,[39,40] we found an $S1P_3$ antagonist did not inhibit HDL-dependent effects in

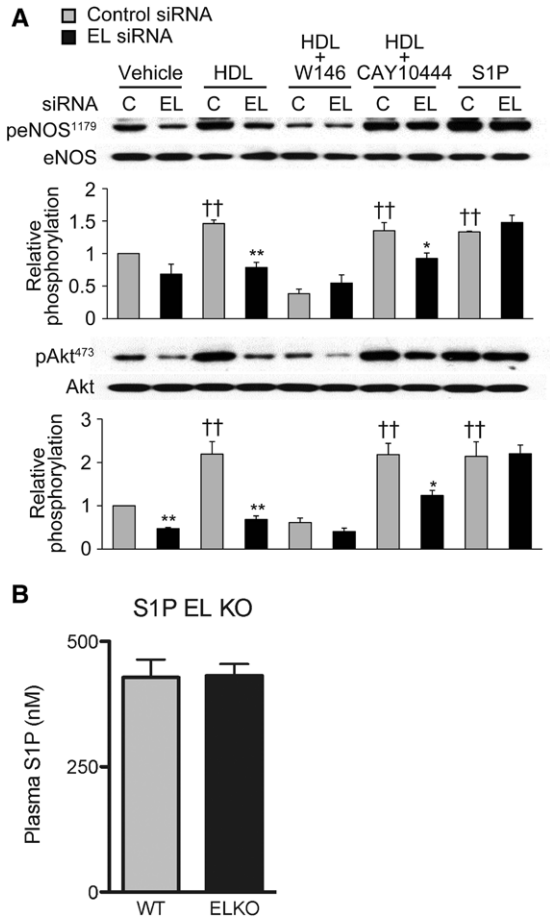


Figure 6. Sphingosine 1-phosphate (S1P)/S1P$_1$ is involved in high-density lipoprotein (HDL)–induced phosphorylation of endothelial NO synthase (eNOS) and Akt mediated by endothelial lipase. **A**, Immunoblots were analyzed in bovine aortic endothelial cells transfected with control or endothelial lipase (EL) small interfering RNA (siRNA) after treatment with vehicle, HDL (75 μg/mL), HDL (75 μg/mL) + W146 (10 μmol/L), HDL (75 μg/mL) + CAY10444 (10 μmol/L), or S1P (100 nmol/L). Vehicle or W146 was added to the cells 1 hour before addition of HDL or S1P. Cell lysates were harvested 5 minutes after adding HDL or S1P and analyzed in immunoblots probed with specific antibodies against Ser^{1179}-phospho-eNOS (peNOS^{1179}), Ser^{473}-phospho-Akt (pAkt^{473}), and total eNOS and Akt. The experiment shown is a representative of 3 independent experiments. **P*<0.05, ***P*<0.01 vs control siRNA with the same treatment, ††*P*<0.01 vs control siRNA with vehicle. Data represent mean±SEM. **B**, S1P plasma levels from wild-type (WT; n=4) and EL-deficient mice (n=6) do not differ on LC/MS/MS measurement (also see Figure II in the online-only Data Supplement).

our system, whereas an S1P$_1$ antagonist completely blocked responses to HDL. Bovine aortic ECs do express both S1P$_3$ and S1P$_1$.[42,43] Our experiments using S1P receptor subtype-selective antagonists identify the S1P$_1$ receptor subtype as the principal receptor mediating the HDL response in these ECs.

Taken together, these findings reveal EL as a key player in facilitating the activation of HDL-dependent signals that lead to S1P$_1$ receptor activation as well as Akt and eNOS phosphorylation, with functional effects on EC migration, tube formation, and angiogenesis (Figure 7). These results provide new insights into the molecular mechanisms through which HDL, EL, and their interaction may modulate angiogenesis and vascular responses.

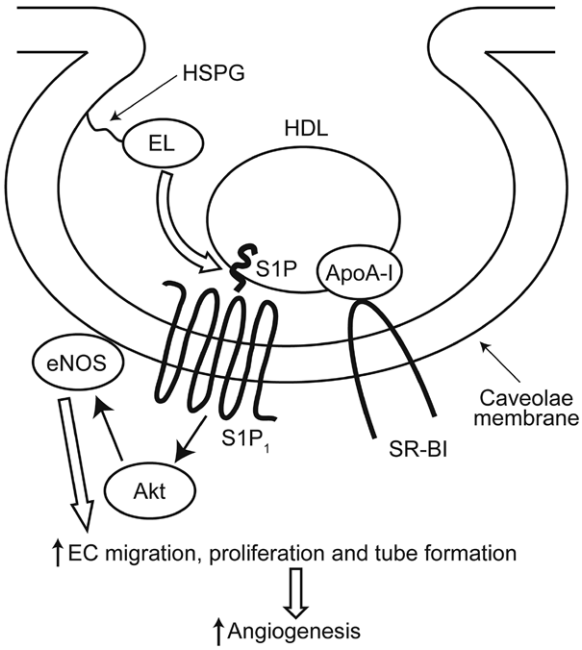


Figure 7. Model of endothelial lipase (EL)–mediated high-density lipoprotein (HDL)–dependent signaling via S1P/S1P$_1$ pathways. The data presented here suggest that HDL hydrolysis by endothelial lipase can release biologically active molecules that promote Akt and endothelial NO synthase (eNOS) phosphorylation in endothelial cells, with functional effects promoting endothelial cell migration, tube formation, and angiogenesis. These EL/HDL responses seem to involve HDL-bound S1P, which then binds to S1P$_1$ receptors, inducing these endothelial effects. Key proteins involved in HDL biology, including SR-B1, ABC1, and ABCG8, have all been implicated in S1P transport. ABCA1 and ABCG1 indicate ATPbinding cassette transporters; ApoA-I. apolipoprotein A-I; HSPG, heparan sulfate proteoglycan; and SR-BI scavenger receptor class B type I.

Acknowledgments

We thank Hong Wang for technical assistance, and Takashi Shiroto and Ruqin Kou for helpful discussions.

Sources of Funding

This study was supported by National Institutes of Health grants HL48743 (T. Michel, J. Plutzky) and HL46457 (T. Michel), and the Mitsubishi Pharma Research Foundation and the Keio University Medical Science Fund (S. Tatematsu).

Disclosures

None.

References

1. Gordon DJ, Rifkind BM. High-density lipoprotein–the clinical implications of recent studies. *N Engl J Med*. 1989;321:1311–1316.
2. Rader DJ, Alexander ET, Weibel GL, Billheimer J, Rothblat GH. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J Lipid Res*. 2009;50 (suppl):S189–S194.
3. Miura S, Fujino M, Matsuo Y, Kawamura A, Tanigawa H, Nishikawa H, Saku K. High density lipoprotein-induced angiogenesis requires the activation of Ras/MAP kinase in human coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol*. 2003;23:802–808.
4. Barter PJ, Nicholls S, Rye K-A, et al. Antiinflammatory properties of HDL. *Circ Res*. 2004;95:764–772.
5. Viswambharan H, Ming X-F, Zhu S, et al. Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/endothelial nitric oxide synthase. *Circ Res*. 2004;94:918–925.

6. Sattler K, Levkau B. Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. *Cardiovasc Res*. 2009;82:201–211.
7. Argraves KM, Argraves WS. HDL serves as a S1P signaling platform mediating a multitude of cardiovascular effects. *J Lipid Res*. 2007;48:2325–2333.
8. Rodriguez C, Gonzalez-Diez M, Badimon L, et al. Sphingosine-1-phosphate: a bioactive lipid that confers high-density lipoprotein with vasculoprotection mediated by nitric oxide and prostacyclin. *Thromb Haemost*. 2009;101:665–673.
9. Rosen H, Gonzalez-Cabrera PJ, Sanna MG, et al. Sphingosine 1-phosphate receptor signaling. *Annu Rev Biochem*. 2009;78:743–768.
10. Lee MJ, Thangada S, Claffey KP, et al. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell*. 1999;99:301–312.
11. Argraves KM, Gazzolo PJ, Groh EM, et al. High density lipoprotein-associated sphingosine 1-phosphate promotes endothelial barrier function. *J Biol Chem*. 2008;283:25074–25081.
12. Nofer JR, van der Giet M, Tölle M, et al. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*. 2004;113:569–581.
13. Hirata K, Dichek HL, Cioffi JA, et al. Cloning of a unique lipase from endothelial cells extends the lipase gene family. *J Biol Chem*. 1999;274:14170–14175.
14. Jaye M, Lynch KJ, Krawiec J, et al. A novel endothelial-derived lipase that modulates HDL metabolism. *Nat Genet*. 1999;21:424–428.
15. Yu KCW, David C, Kadambi S, et al. Endothelial lipase is synthesized by hepatic and aorta endothelial cells and its expression is altered in apoE-deficient mice. *J Lipid Res*. 2004;45:1614–1623.
16. McCoy MG, Sun G-S, Marchadier D, et al. Characterization of the lipolytic activity of endothelial lipase. *J Lipid Res*. 2002;43:921–929.
17. Ishida T, Choi S, Kundu RK, et al. Endothelial lipase is a major determinant of HDL level. *J Clin Invest*. 2003;111:347–355.
18. Edmondson AC, Brown RJ, Kathiresan S, et al. Loss-of-function variants in endothelial lipase are a cause of elevated HDL cholesterol in humans. *J Clin Invest*. 2009;119:1042–1050.
19. Brown RJ, Edmondson AC, Griffon N, et al. A naturally occurring variant of endothelial lipase associated with elevated HDL exhibits impaired synthesis. *J Lipid Res*. 2009;50:1910–1916.
20. Ishida T, Choi SY, Kundu RK, et al. Endothelial lipase modulates susceptibility to atherosclerosis in apolipoprotein-E-deficient mice. *J Biol Chem*. 2004;279:45085–45092.
21. Ko KWS, Paul A, Ma K, et al. Endothelial lipase modulates HDL but has no effect on atherosclerosis development in apoE-/- and LDLR-/- mice. *J Lipid Res*. 2005;46:2586–2594.
22. Broedl UC, Maugeais C, Millar JS, et al. Endothelial lipase promotes the catabolism of ApoB-containing lipoproteins. *Circ Res*. 2004;94:1554–1561.
23. Ahmed W, Orasanu G, Nehra V, et al. High-density lipoprotein hydrolysis by endothelial lipase activates PPARalpha: a candidate mechanism for high-density lipoprotein-mediated repression of leukocyte adhesion. *Circ Res*. 2006;98:490–498.
24. Vergeer M, Cohn DM, Boekholdt SM, et al. Lack of association between common genetic variation in endothelial lipase (LIPG) and the risk for CAD and DVT. *Atherosclerosis*. 2010;211:558–564.
25. Voight BF, Peloso GM, Orho-Melander M, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet*. 2012;380:572–580.
26. Skoura A, Hla T. Regulation of vascular physiology and pathology by the S1P2 receptor subtype. *Cardiovasc Res*. 2009;82:221–228.
27. Igarashi J, Michel T. Sphingosine 1-phosphate and isoform-specific activation of phosphoinositide 3-kinase beta. Evidence for divergence and convergence of receptor-regulated endothelial nitric-oxide synthase signaling pathways. *J Biol Chem*. 2001;276:36281–36288.
28. Sevvana M, Ahnstrom J, Egerer-Sieber C, et al. Serendipitous fatty acid binding reveals the structural determinants for ligand recognition in apolipoprotein M. *J Mol Biol*. 2009;393:920–936.
29. Christoffersen C, Obinata H, Kumaraswamy SB, Galvani S, Ahnström J, Sevvana M, Egerer-Sieber C, Muller YA, Hla T, Nielsen LB, Dahlbäck B. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. *Proc Natl Acad Sci U S A*. 2011;108:9613–9618.
30. Saddar S, Mineo C, Shaul PW. Signaling by the high-affinity HDL receptor scavenger receptor B type I. *Arterioscler Thromb Vasc Biol*. 2010;30:144–150.
31. O'Connell BJ, Genest J Jr. High-density lipoproteins and endothelial function. *Circulation*. 2001;104:1978–1983.
32. Kimura T, Tomura H, Sato K, Ito M, Matsuoka I, Im DS, Kuwabara A, Mogi C, Itoh H, Kurose H, Murakami M, Okajima F. Mechanism and role of high density lipoprotein-induced activation of AMP-activated protein kinase in endothelial cells. *J Biol Chem*. 2010;285:4387–4397.
33. Drew BG, Fidge NH, Gallon-Beaumier G, Kemp BE, Kingwell BA. High-density lipoprotein and apolipoprotein AI increase endothelial NO synthase activity by protein association and multisite phosphorylation. *Proc Natl Acad Sci U S A*. 2004;101:6999–7004.
34. Hara T, Ishida T, Kojima Y, Tanaka H, Yasuda T, Shinohara M, Toh R, Hirata K. Targeted deletion of endothelial lipase increases HDL particles with anti-inflammatory properties both *in vitro* and in vivo. *J Lipid Res*. 2011;52:57–67.
35. Riederer M, Lechleitner M, Hrzenjak A, Koefeler H, Desoye G, Heineman A, Frank S. Endothelial lipase (EL) and EL-generated lysophosphatidylcholines promote IL-8 expression in endothelial cells. *Atherosclerosis*. 2011;214:338–344.
36. Khurana R, Simons M, Martin JF, Zachary IC. Role of angiogenesis in cardiovascular disease: a critical appraisal. *Circulation*. 2005;112:1813–1824.
37. Igarashi J, Bernier SG, Michel T. Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase. differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. *J Biol Chem*. 2001;276:12420–12426.
38. Kontush A, Therond P, Zerrad A, Couturier M, Négre-Salvayre A, de Souza JA, Chantepie S, Chapman MJ. Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL3 particles: relevance to antiapoptotic and antioxidative activities. *Arterioscler Thromb Vasc Biol*. 2007;27:1843–1849.
39. Liu X, Xiong SL, Yi GH. ABCA1, ABCG1, and SR-BI: Transit of HDL-associated sphingosine-1-phosphate. *Clin Chim Acta*. 2012;413:384–390.
40. Igarashi J, Miyoshi M, Hashimoto T, Kubota Y, Kosaka H. Statins induce S1P1 receptors and enhance endothelial nitric oxide production in response to high-density lipoproteins. *Br J Pharmacol*. 2007;150:470–479.
41. Karuna R, Park R, Othman A, Holleboom AG, Motazacker MM, Sutter I, Kuivenhoven JA, Rohrer L, Matile H, Hornemann T, Stoffel M, Rentsch KM, von Eckardstein A. Plasma levels of sphingosine-1-phosphate and apolipoprotein M in patients with monogenic disorders of HDL metabolism. *Atherosclerosis*. 2011;219:855–863.
42. Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S, Spiegel S. Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. *J Biol Chem*. 1999;274:35343–35350.
43. Lee H, Goetzl EJ, An S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol*. 2000;278:C612–C618.

Significance

Controversies persist about the relationship between high-density lipoprotein (HDL) and atherosclerosis. Despite a well-validated inverse relationship between HDL levels and cardiovascular events in epidemiological studies, HDL-raising therapies levels have not shown clear clinical benefit in prospective, randomized clinical trials. Moreover, recent data reveal that genetic loss-of-function endothelial lipase (EL) variants have increased HDL levels without any obvious protection against cardiovascular events. Extensive studies now establish that specific lipases can exert many distinct and important biological effects. We provide evidence here that EL interacts with HDL to promote angiogenesis, increase endothelial proliferation, and endothelial cell signaling via endothelial NO synthase and aKT phosphorylation. These EL/HDL seem to involve the release of sphingosine-1 phosphate and signaling through the $S1P_1$ receptor. These data provide new perspectives on how EL interaction with HDL may modulate endothelial responses through S1P release and offer an example of how EL deficiency might result in a loss of potentially beneficial effects.

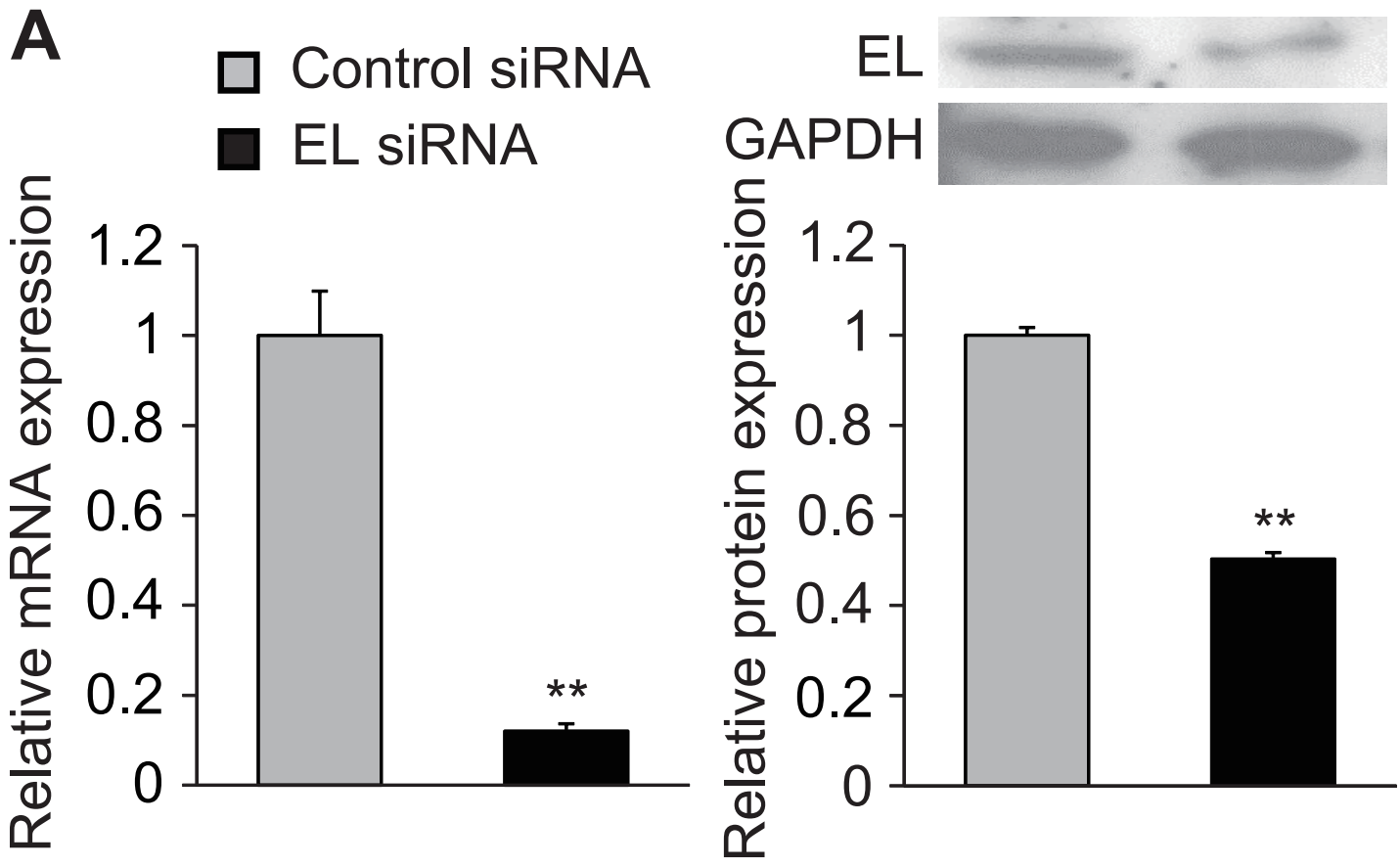
Supplement Material

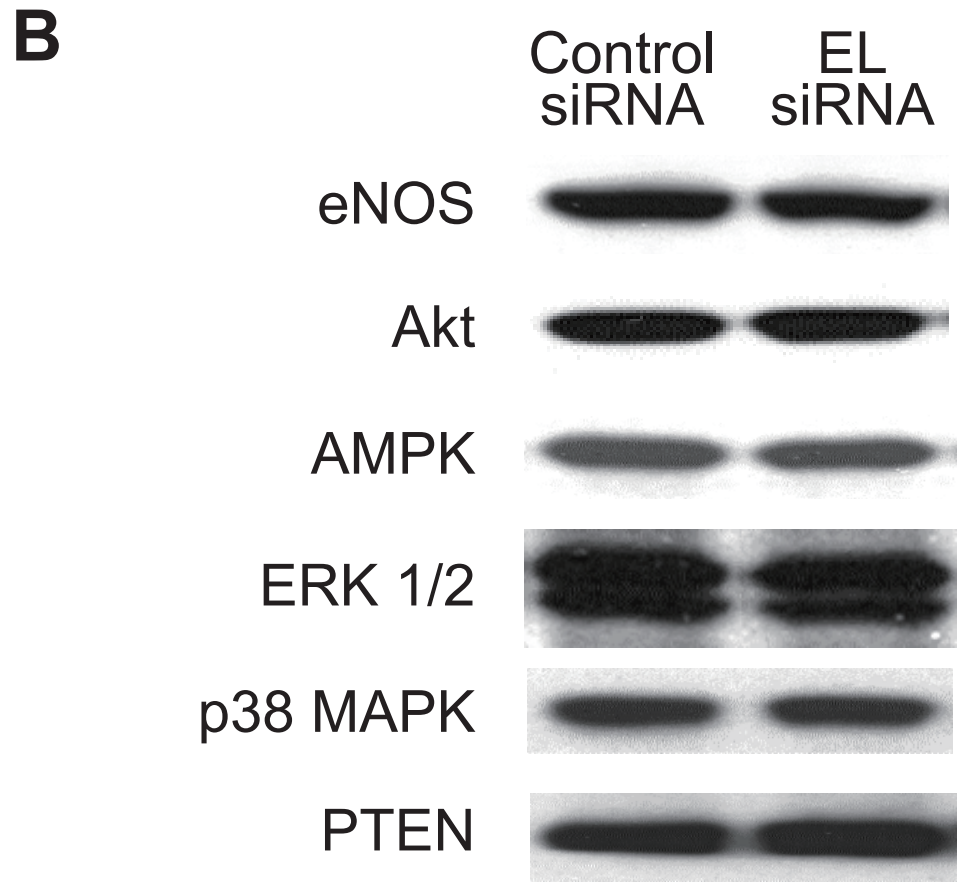
Supplement Figure Legends

Supplement Figure I. siRNA to Endothelial Lipase (EL) Decreased Expression and Protein levels of EL But Not Other Relevant EC Targets. A. EL siRNA treatment as outlined in Methods significantly decreased EL mRNA (90%, left panel) and protein (50%, right panel) levels as compared to control siRNA, as seen on RT-PCR and Western blotting. B. Similar EL and control siRNA EC treatment protocols as employed in 1A and throughout the manuscript did not alter mRNA or protein levels of eNOS, Akt, AMPK, ERK1/2, p38 MAPK, or PTEN (abbreviations as per manuscript).

Supplement Figure II. S1P Chromatograms. A. Endogenous and Exogenous, C17-labeled S1P Detection In Murine Plasma. HPLC tandem MS was performed on murine plasma from wildtype mice to which C17-labelled S1P had been added, yielding a precursor ion m/z 364 (C17 standard) and the product ion m/z 79 (phosphate head group). Comparison to wildtype plasma without the addition of exogenous S1P demonstrated a precursor ion m/z 378.2 with the expected product ion (m/z 79). B. S1P chromatograms on plasma from wildtype mice (n = 4). C. S1P chromatograms on plasma from EL-deficient mice (n = 6). The upper number on the S1P peak represents quantification of the S1P detected.

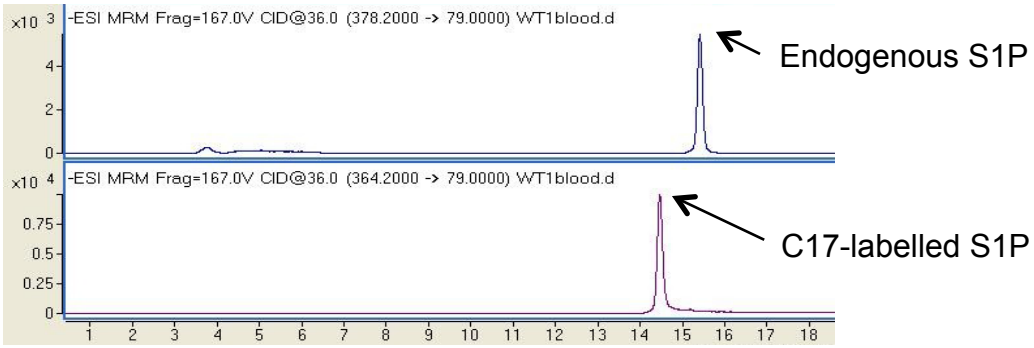
Supplement Figure I



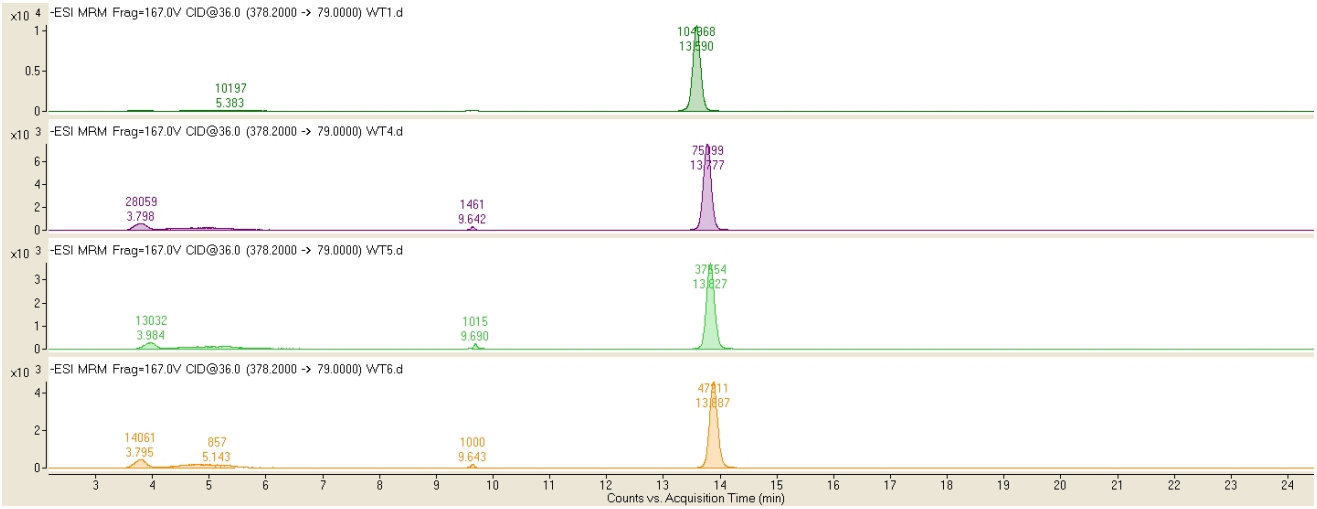


Supplement Figure II

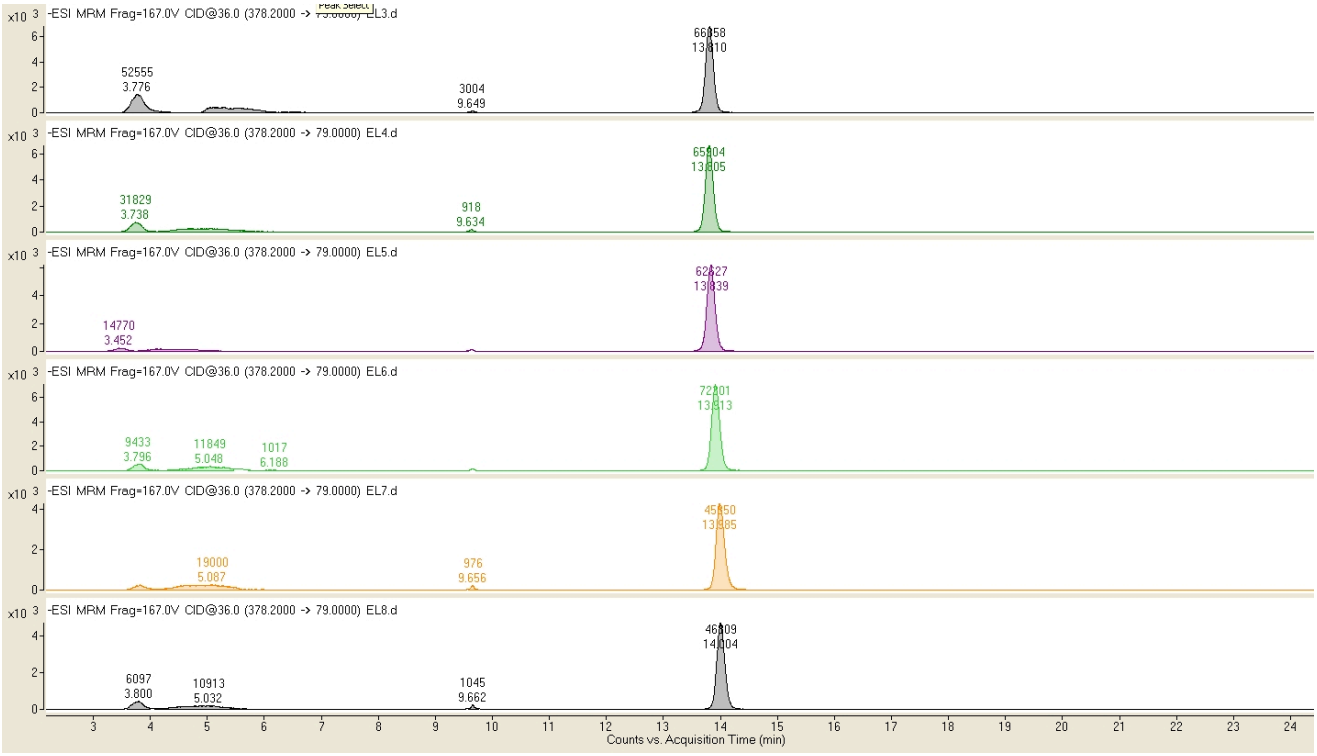
A. Chromatograms of wildtype murine plasma with and without addition of C17-labelled S1P



B. S1P Chromatograms of plasma samples from wildtype mice



C. S1P Chromatograms of plasma samples from EL-deficient mice



Methods

Reagents

Tetrahydrolipstatin was obtained from Roche Laboratories (Nutley, NJ). Pooled normal human HDL and LDL were from Sigma-Aldrich and Biomedical Technologies respectively (Stoughton, MA). Mouse anti-endothelial nitric oxide synthase (eNOS) and mouse anti-phospho-eNOS (Ser^{1177}) antibodies were from BD Biosciences (San Diego, CA). Rabbit polyclonal antibodies against Akt and phospho-Akt (Ser^{473}) were from Cell Signaling Technology (Danvers, MA). W146 and S1P were from Avanti Polar Lipids (Alabaster, AL). CAY10444 was from Cayman Chemical (Ann Arbor, MI). All other reagents were from Sigma (St. Louis, MO).

Cell culture

Bovine aortic endothelial cells (BAEC) were obtained from Cambrex (Walkersville, MD), grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine, penicillin, streptomycin, and fungizone, and studied between passages 6 and 8 as before.[1, 2]

siRNA transfection

Small interfering RNA (siRNA, Ambion, Austin, TX) against endothelial lipase was 5'-ACGUGACAGCCAAAACCUUtt-3' and the negative control sequence was: 5'-AUUGUAUGCGAUCGCAGACtt-3'. BAEC were transfected with siRNA when cells were ~50–70% confluence. Transfected cells were maintained in antibiotic-free DMEM/10% FBS. Transfection with siRNA (30 nM) was done using Lipofectamine 2000 (0.15% vol/vol) following the manufacturer's protocol (Invitrogen, Carlsbad, CA) in media supplemented with 10% FBS 5 h after transfection; experiments were conducted 48 h after transfection.

Endothelial cell migration and proliferation assay

To assess the effect of HDL and EL on cell migration, co-culture inserts from Culture-Inserts (Ibidi, Madison, WI) were used. BAEC (5 x 10^5 cells/ml, 70 μl) were seeded in two cell culture reservoirs separated by a silicon insert. HDL concentrations chosen were based on prior published reports [3] and concentration-ranging studies performed here. After 18 h, the cells were washed with phosphate buffered solution (PBS) prior to serum starvation in serum-free DMEM containing .1% bovine serum albumin and no other mitogens. After 6 h, the inserts were removed, the cells were rinsed twice with PBS, and the culture medium replaced by fresh medium with vehicle, HDL (75 μg/ml), W146 (10 μM), CAY10444 (10 μM) or S1P (100 nM). Cell images were obtained using an inverted microscope (ECLIPSE TE 2000-U, Nikon, Tokyo, Japan) 12 h after insert removal and migration quantified using Image-Pro Plus software. The cells were incubated for 24 h, then trypsinized and counted using a hemocytometer to assess cell proliferation.

Cell viability assay

Cell viability was determined by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, final concentration 0.5 mg/ml). BAEC were plated in a 96-well plate at a density of 3×10^4 cells per well in 10% FBS media for 24 h. Three duplicate wells were set up in each sample. At least three independent experiments were carried out. The cells were incubated with MTT (4 h, 37ºC). The plate was gently agitated until the color reaction was uniform; OD_{570} nm was determined by microplate reader (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA). Results shown are an average fold-change of cell viability relative to the control (control siRNA in vehicle).

Tube formation assay

24-well plates were coated with Matrigel (BD Biosciences, San Diego, CA, 250 μl/well) and incubated (30 min, 37ºC) for gel polymerization. Forty-eight hours after siRNA transfection, BAEC were trypsinized and re-plated on Matrigel-treated wells at a density of 3 X 10^4 cells/well in serum-free DMEM (250 μl) with vehicle or HDL (75 μg/ml). The cells were incubated (9 h, 37ºC, 5% CO_2 incubator) and imaged using phase-contrast microscopy (ECLIPSE TE 2000-U, Nikon). Four random fields of view (FOV)/well were examined and photographed by a blinded observer. For quantification, a node was defined as an aggregation of cells from which three or more tube-like structures originated; a tube was defined as a continuous stretch of at least two cells containing no more than two nodes. The total tube length was measured using Image-Pro Plus software. Each experimental arm was repeated in five wells.

Mice

Male homozygous EL-/- mice, which were backcrossed with the C57BL6/J strain for at least 12 generations, and age-matched C57BL6/J wild-type (WT) mice were used in this study as described previously, including 12-h light/dark cycle housing with food and water *ad libitum*.[4] All experimental procedures were conducted according to Harvard Medical School Institutional Animal Care and Use Committee protocols.

Aortic ring angiogenesis assay

Aortic ring angiogenesis assays were performed using thoracic aortas removed from 1–2 month old mice (WT and $EL^{-/-}$), following established methods.[5, 6] Aortas were transferred into a dish containing sterilized cold PBS. Peri-aortic fibro-adipose tissues were carefully removed under surgical microscopy, the aorta cut into rings (1 mm wide), and washed 5 times (Medium 199, Lonza, Allendale, NJ). A 96-well plate was coated with Matrigel (50 μl, BD Biosciences, San Diego, CA). After polymerization (37ºC), the aortic rings were placed in the wells and

covered with Matrigel (50 μl). After polymerization (30 min, 37ºC), 100 μl RPMI 1640 (Lonza, Walkersville, MD) was added to each well, either with or without HDL (75 μg/ml). After 4-5 days of culture, the aortas were photographed with a phase contrast microscope by a blinded observer. The area of EC outgrowth was analyzed using Image-Pro Plus software.

Western blot analysis

Western blotting was performed as before.[7] For cell extraction, cells were lysed in immunoprecipitation buffer (Boston BioProducts, Ashland, MA) containing protease and phosphatase inhibitor cocktails (both Roche Diagnostics, Indianapolis, IN). Proteins from whole cell lysates of BAEC were separated on 4-12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene fluoride membrane (Perkin Elmer, Waltham, MA). After transfer, the membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 (1 h), probed with primary antibodies and washed (TBS-Tween20, 15 min, three times). The membranes were probed with secondary antibodies for 1 h and washed three times. The signal was detected using enhanced chemiluminescence (Perkin Elmer, Waltham, MA) using manufacturer's protocols.

mRNA isolation and real-time quantitative PCR (RT-qPCR)

RNA was isolated from BAEC using RNeasy (Qiagen, Valencia, CA) and quantified using NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE). cDNA was prepared using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed using iQ SYBR Green Supermix and iCycler Real-Time PCR Detection System (Bio-Rad). The sequences used for PCR were as follows: *LIPG*, 5'-CAGAGGATGAAGGATGCGATC-3' for forward primer and 5'-TGACACCAGTTTGTACAGCC-3' for reverse primer; *ACTB*, 5'-GGCACCCAGCACAATGAAGATCAA-3' for forward primer and 5'-

ATCGTACTCCTGCTTGCTGATCCA-3' for reverse primer. mRNA levels were normalized to β-actin as internal control.

Mass Spectroscopy for S1P Quantification

LC/MS/MS analysis was performed using an Agilent 6410 triple quadrupole mass spectrometer in negative ionization mode with an electrospray source ionization interface, adapting prior methods[8]. For LC analysis a Gemini (Phenomenex) C18 column (50 mm x 2.1 mm, 3 μm particle size, 100 angstrom pore) was used with a 50 μm steel mesh filter. Mobile phase A consisted of 95/5 water/methanol, mobile phase B 65/35/5 isopropanol/methanol/water, both supplemented with 0.1% ammonium hydroxide. The flow rate was 0.3 μl/min. The gradient started at 20% B and linearly increased to 100% B (45 minutes), was maintained at 100% B (10 minutes) before equilibrating at 20% B (5 minutes). MS was operated in multiple reaction monitoring (MRM) mode, monitoring for S1P using the precursor ion m/z 378.2 to 79 (phosphate head group) transition $[M - H]^+$. For the C17-S1P standard, the m/z transition was 364.2 to 79. Capillary voltage was set to 3.0 kV, the fragmentor voltage to 200 V with a collision energy of 35 V, with drying gas temperature 350 ºC, drying gas flow 10 L/min and nebulizer pressure was 45 psi. Plasma (80 μl) from wildtype (n = 3) and EL-deficient mice (n = 6) was extracted (modified Folch method) and aliquots injected and compared to control samples spiked with a C17-labelled S1P standard. Sensitivity for S1P measurement was in the high femtomole range. The integrated S1P peak area was normalized to the C17 standard peak. Results presented as mean ± SEM.

Statistical Analysis

Data are expressed as mean ± SEM. Results were analyzed by unpaired Student's *t* test or 1-way ANOVA, as appropriate, followed by Newman-Keuls post hoc test. A p value < 0.05 was considered statistically significant.

References for Material/Methods

1. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an antiinflammatory role for lipoprotein lipase. *Proc Natl Acad Sci U S A.* 2003;100:2730-2735.
2. Ahmed W, Orasanu G, Nehra V, Asatryan L, Rader DJ, Ziouzenkova O, Plutzky J. High-density lipoprotein hydrolysis by endothelial lipase activates PPARalpha: a candidate mechanism for high-density lipoprotein-mediated repression of leukocyte adhesion. *Circ Res.* 2006;98:490-498.
3. Miura S-i, Fujino M, Matsuo Y, Kawamura A, Tanigawa H, Nishikawa H, Saku K. High density lipoprotein-induced angiogenesis requires the activation of Ras/MAP kinase in human coronary artery endothelial cells. *Arterioscler Thromb Vasc Biol.* 2003;23:802-808.
4. Ishida T, Choi S, Kundu RK, Hirata K-I, Rubin EM, Cooper AD, Quertermous T. Endothelial lipase is a major determinant of HDL level. *J Clin Invest.* 2003;111:347-355.
5. Reed MJ, Karres N, Eyman D, Vernon RB. Culture of murine aortic explants in 3-dimensional extracellular matrix: a novel, miniaturized assay of angiogenesis in vitro. *Microvasc Res.* 2007;73:248-252.
6. Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P, Sun C, Zhang Y, Liu J, Ennis TL, Knispel R, Xiong W, Thompson RW, Baxter BT, Shi GP. Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice. *J Clin Invest.* 2007;117:3359-3368.
7. Kanda T, Brown JD, Orasanu G, Vogel S, Gonzalez FJ, Sartoretto J, Michel T, Plutzky J. PPARgamma in the endothelium regulates metabolic responses to high-fat diet in mice. *J Clin Invest.* 2009;119:110-124.
8. Brown JD, Oligino E, Rader DJ, Saghatelian A, Plutzky J. VLDL hydrolysis by hepatic lipase regulates PPARdelta transcriptional responses. *PLoS One.* 2011;6:e21209.