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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 receptors and promotes activation of endothelial nitric oxide 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 eNOS179 and Akt473. 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 S1P1 antagonist W146 but not by the S1P3 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 S1P1, 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
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 al22 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 al24 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 20913 myocardial infarction cases compared with 95407 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 S1P1 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 EC50 for S1P1 receptor subtype-selective antagonists to assess the role of EL in HDL-induced EC migration. Addition of HDL (100 μg/mL) approximately doubled EC migration as compared with vehicle stimulation, an effect blocked by tetrahydrolipstatin (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 Ser1179 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/S1P1
SIP1 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 S1P1 antagonist W146 blocked HDL-induced EC migration and Akt/eNOS phosphorylation. In contrast, the

Materials and Methods
Materials and Methods are available in the online-only Supplement.
S1P, 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, but not S1P3, 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. 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, whereas Akt-dependent...
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 al34 reported that HDL from EL knockout mice retained the anti-inflammatory features seen in HDL isolated from WT mice. Moreover, Riederer et al35 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 S1P1 receptor antagonist W146 blocked HDL-induced EC migration as well as Akt/eNOS phosphorylation, whereas the S1P3 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.11,12,25 We previously reported that free S1P activates eNOS through phosphoinositide 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...
highly enriched in S1P relative to large HDL2 particles. 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, might be involved in the EL-mediated effects reported here. One recent study analyzed S1P plasma levels in relationship to apolipoprotein M in different monogenic disorders of HDL metabolism. 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 heterogeneous 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), 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 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.

We also provide evidence that S1P released from HDL by EL activates S1P1 receptors that promote Akt/eNOS activation. Although other model systems have implicated both S1P1 and S1P3 receptors in these responses, we found an S1P3 antagonist did not inhibit HDL-dependent effects in analyzed S1P plasma levels in relationship to apolipoprotein M in different monogenic disorders of HDL metabolism.

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our system, whereas an S1P1 antagonist completely blocked responses to HDL. Bovine aortic endothelial cells transfected with control or endothelial lipase (EL) small interfering RNA (siRNA) and Akt mediated by endothelial lipase. 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 Ser1179-phospho-eNOS (pE1179), Ser473-phospho-Akt (pAkt473), 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).

Figure 7. Model of endothelial lipase (EL)-mediated high-density lipoprotein (HDL)-dependent signaling via S1P/S1P1 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 S1P1 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 ATP-binding cassette transporters; ApoA-I, apolipoprotein A-I; HSPG, heparan sulfate proteoglycan; and SR-BI scavenger receptor class B type I.

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Disclosures
None.

References
angiogenesis, increase endothelial proliferation, and endothelial cell signaling via endothelial NO synthase and aKT phosphorylation. These specific lipases can exert many distinct and important biological effects. We provide evidence here that EL interacts with HDL to promote clinical benefit in prospective, randomized clinical trials. Moreover, recent data reveal that genetic loss-of-function endothelial lipase (EL) relationship between HDL levels and cardiovascular events in epidemiological studies, HDL-raising therapies levels have not shown clear Controversies persist about the relationship between high-density lipoprotein (HDL) and atherosclerosis. Despite a well-validated inverse

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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 SIP receptor. These data provide new perspectives on how EL interaction with HDL may modulate endothelial responses through SIP release and offer an example of how EL deficiency might result in a loss of potentially beneficial effects.


20. Kountou A, Therond P, Zerrad A, Couturié M, Négre-Salvayre A, de Souza IA, 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 activi-


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

A

Control siRNA
EL siRNA

Relative mRNA expression

0.2 0.4 0.6 0.8 1 1.2

Relative protein expression

0.2 0.4 0.6 0.8 1 1.2

EL
GAPDH

Control siRNA
EL siRNA

B

eNOS
Akt
AMPK
ERK 1/2
p38 MAPK
PTEN

**
Supplement Figure II

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

- Endogenous S1P
- 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\textsuperscript{1177}) antibodies were from BD Biosciences (San Diego, CA). Rabbit polyclonal antibodies against Akt and phospho-Akt (Ser\textsuperscript{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.\textsuperscript{1,2}

siRNA transfection

Small interfering RNA (siRNA, Ambion, Austin, TX) against endothelial lipase was 5’-ACGUGACAGCCAAAACCUCUtt-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 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 x 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, 37°C). 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, 37°C) 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 10⁴ cells/well in serum-free DMEM (250 µl) with vehicle or HDL (75 µg/ml). The cells were incubated (9 h, 37°C, 5% CO₂ 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 (37°C), the aortic rings were placed in the wells and
covered with Matrigel (50 µl). After polymerization (30 min, 37°C), 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. 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’- CAGAGGATGAGGATGCGAT-3’ for forward primer and 5’- TGACACCAGTTGTACAGCC-3’ for reverse primer; *ACTB*, 5’- GGCACCCAGCAATGAAGATCAA-3’ for forward primer and 5’-
ATCGTACTCCTGCTTGCTGAC 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. 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


