Endothelial sphingolipid *de novo* synthesis controls blood pressure by regulating signal transduction and NO via ceramide

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Abstract

Ceramides are sphingolipids that modulate a variety of cellular processes via two major mechanisms: functioning as second messengers and regulating membrane biophysical properties, particularly lipid rafts, important signaling platforms. Altered sphingolipid levels have been implicated in many cardiovascular diseases, including hypertension, atherosclerosis and diabetes-related conditions, however molecular mechanisms by which ceramides impact endothelial functions remain poorly understood. In this regard, we generated mice defective of endothelial sphingolipid *de novo* biosynthesis by deleting the long chain subunit 2 of serine palmitoyltransferase, the first enzyme of the pathway.

Our study demonstrated that endothelial sphingolipid *de novo* production is necessary to regulate 1) signal transduction in response to NO agonists and, mainly via ceramides; 2) resting eNOS phosphorylation; and 3) blood pressure homeostasis. Specifically, our findings suggest a prevailing role of C16:0-ceramide in preserving vasodilation induced by tyrosine kinase and G-protein coupled receptors, except for Gq-coupled receptors, while C24:0- and C24:1-ceramides control flow-induced vasodilation. Replenishing C16:0-ceramide *in vitro* and *in vivo* reinstates endothelial cell signaling and vascular tone regulation.

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This study reveals an important role of locally produced ceramides, particularly C16:0-, C24:0- and C24:1-ceramides in vascular and blood pressure homeostasis, and establishes the endothelium as a key source of plasma ceramides. Clinically, specific plasma ceramides ratios are independent predictors of major cardiovascular events. Our data also suggest that plasma ceramides might be indicative of the "diseased" state of the endothelium.

Graphical Abstract
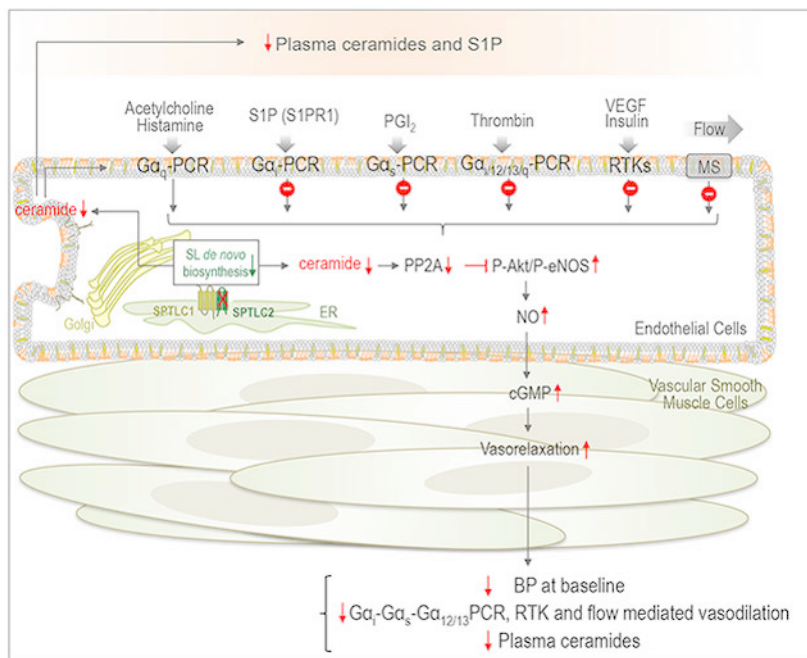
Summary:

Endothelial *de novo* biosynthesis of sphingolipids is necessary to preserve vascular functions and BP homeostasis

Keywords

Sphingolipid metabolism; ceramides; endothelial function; vascular tone regulation; blood pressure

INTRODUCTION

The endothelium regulates vascular and blood pressure (BP) homeostasis by integrating moment-to-moment chemical and rheological stimuli, and the loss of this function is an early event in the pathogenesis of many cardiovascular conditions[1-4].

Recently, ceramides, a subclass of sphingolipids, have been implicated in endothelial dysfunction during obesity, diabetes and their cardiovascular complications[5-8].

Ceramide is the precursor of all major complex sphingolipids, important membrane components. Metabolically, ceramide is generated by the sphingolipid *de novo* biosynthesis as well as by the breakdown of complex sphingolipids, including sphingomyelin and glycosphingolipids[9]. During stress responses, the activation of either or both pathways[10, 11] leads to cellular accumulation of ceramides. Additionally, ceramide can be formed by the salvage pathway[12], which re-acylates sphingosine derived from sphingolipid catabolism.

The hydrophobicity of ceramide can alter biophysical membrane properties, particularly of lipid rafts, specialized signaling platforms[13-15]. Ceramide is also a bioactive lipid able to regulate multiple biological processes including stress responses and apoptosis, in part, by the activation of different targets, such as protein phosphatase PP2A and PP1 [16, 17], protein kinase Cζ activity[18] and cathepsin D[19].

Recent studies suggested that specific plasma ceramide ratios are independent predictors of major cardiovascular events[5, 20]. During metabolic syndrome and type 2 diabetes, ceramides have been implicated in endothelial dysfunction via PP2A-mediated dephosphorylation of eNOS[21, 22]. While mechanisms of ceramide-mediated apoptosis have been exploited particularly in cancer, how ceramides regulate vascular functions and BP is poorly understood.

The SL *de novo* biosynthesis is initiated by serine palmitoyltransferase (SPT)[23, 24], the first and rate-limiting enzyme. Mice lacking SPT are embryonically lethal[25], emphasizing the requirement of SL for survival. Recently, we identified Nogo-B, a membrane protein of the endoplasmic reticulum, as a negative regulator of SPT activity [26]. Mice lacking Nogo-B, systemically or in the endothelium, are resistant to hypertension[26], inflammation[27, 28] and heart failure[27], suggesting that the decrease of SPT activity by Nogo-B has pathological implications.

To directly investigate the impact of the local SL *de novo* biosynthesis on vascular functions and BP, we generated mice lacking endothelial SPT long chain subunit 2 (*Sptlc2*), one of the two subunits forming SPT[32, 33]. Our studies demonstrate that SL *de novo* biosynthesis is necessary to maintain vascular and BP homeostasis. Mechanistically, the decrease of ceramides while impairs endothelial signal transduction in response to chemical and rheological stimuli, enhances basal eNOS phosphorylation state, and thus NO production. Among the most abundant endothelial ceramides, C16:0-Cer preserves endothelial receptor-mediated signaling, while C24:0-Cer and C24:1-Cer mainly control flow-mediated vasodilation. Furthermore, our study shows that plasma ceramides are dictated by the endothelium, thus circulating ceramides may be a "raconteur" of the health/diseases state of the vasculature.

METHODS AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon reasonable request

Mouse model.

We generated conditional mouse model lacking endothelial SPTLC2 subunit of SPT specifically, namely ECKO-Sptlc2. Floxed-Sptlc2 mice (Sptlc2$^{f/f}$) were crossed with transgenic mice in which the VE-cadherin promoter drives expression of tamoxifen-responsive Cre (VE-Cad-CreERT2), such that tamoxifen treatment selectively deletes the *lox*P-flanked ('floxed') region of Sptlc2 in EC[29]. To delete Sptlc2 in EC, 7 to 8 weeks old male mice were injected intraperitoneally (i.p.) with 20 mg/kg of tamoxifen daily for 5 consecutive days. All animal experiments were approved by the Weill Cornell Institutional Animal Care and Use Committee.

Statistical analysis.

Data are expressed as mean ± SEM. Two-way ANOVA followed by Sidak's post-test, One-way ANOVA followed by Tukey's post-test, or Student's *t*-test were used for statistical analysis as indicated. Differences were considered statistically significant when p 0.05. All tests were two-sided. GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA) was used for all statistical analysis.

RESULTS

Endothelial sphingolipid *de novo* biosynthesis is an important source of local and circulating ceramide and S1P.

The endothelial deletion of S*ptlc2* gene was obtained by crossing floxed Sptlc2 mice with tamoxifen inducible VE-cadherin Cre recombinase mice(*Sptlc2$^{f/f}$* Cdh5-Cre^{ERT2}; referred to as ECKO-Sptlc2). At 3 weeks post-tamoxifen treatment, the efficiency of Sptlc2 deletion in aortic EC was 80% (Fig. S1). Endothelial SPTLC2 expression and SPT activity were significantly reduced (Fig. 1A, B), as well as ceramides and sphingosine (Fig. 1C, D). Interestingly, whereas plasma ceramides and S1P significantly decreased (~50% ECKO-Sptlc2 vs. controls, Fig. 1E,F), sphingomyelin levels were unchanged suggesting that the endothelium is a critical source of plasma ceramides and S1P but not sphingomyelin (Fig. S1E, F).

Endothelial sphingolipid *de novo* biosynthesis is necessary to preserve vascular tone and BP homeostasis.

To study the impact of endothelial SL changes, a systematic analysis of vascular reactivity in response to NO-agonists as well as flow was performed.

Vasodilations induced by acetylcholine (Ach) and histamine, both mediated by G$\alpha_{q/11}$-coupled receptors[31], were preserved in ECKO-Sptlc2 MA vs. controls (Fig. 2A and Fig. S2A). Interestingly, although basal eNOS phosphorylation (Fig. S4), eNOS-derived NO, plasma nitrites levels, and VASP phosphorylation (Fig. 2A-C) were significantly increased in absence of SPTLC2, there were no changes in stimulated NO production (Fig. 2A), SNP-mediated relaxation (Fig. S2C), and in iNOS and nNOS levels (Fig. S2D).

On the contrary, the vasorelaxation in response to other agonists activating receptors other than Gq-CPR, such as S1P receptor-1 (S1P1), coupled to Gα_i proteins[32]; protease-activated-

receptor-1 (PAR1), coupled with $G\alpha_{12/13}$, $G\alpha_q$ and $G\alpha_i$[33]; and PGI_2 receptor (IP)[34], preferentially coupled to $G\alpha_s$[35] and VEGFR2[36], were all significantly reduced in ECKO-Sptlc2 MA compared to control (Fig. 2D,E). Insulin-mediated vasodilation[37] was also reduced in Sptlc2-KO MA (Fig. S2B), suggesting that SL are generally required for receptor tyrosine kinase (RTK) signaling. Furthermore, the loss of SPTLC2 markedly suppressed flow-mediated vasodilation (Fig. 2F), whereas vasoconstriction by phenylephrine and U46619 (thromboxane A2 receptor agonist) were preserved (Fig. S2E, F). Overall, the decrease of local SL production heightens basal NO, while impairs endothelial cell signaling in response to physical and chemical stimuli, contributing to vascular tone dysregulation.

At the same time point, we measured BP by using radiotelemetry. Systolic, diastolic and mean BP, but not heart rate, were significantly reduced in ECKO-Sptlc2 compared to Sptcl2$^{f/f}$ (Fig. 3A-D). Furthermore, there were no differences in LF/HF ratios during the light and dark cycles, suggesting a similar sympathetic outflow in ECKO-Sptlc2 compared to Sptlc2$^{f/f}$ mice (Fig. 3E). These findings suggest that the increase in basal NO production might contribute, at least in part, to the lower BP.

Prevailing role of C16:0-Cer over C24:0- and C24:1-Cer in preserving endothelial-mediated vasodilation.

We sought to identify the biological functions of the most abundant endothelial ceramides (Fig. 1E,F), chosen based on the length of the acyl chain (C16:0-Cer vs. C24:0-Cer) and the double bond (C24:1-Cer vs C24:0-Cer). To this end, ECKO-Sptlc2 mice were treated with C16:0-, C24:0- and C24:1-Cer, at two different doses (3 and 10 mg/Kg/d for 2 days).

At low dose C16:0-Cer fully restored and enhanced VEGF-induced vasodilation of ECKO-Sptlc2 vs. control MA (Fig.4A, **left panel**), but not at higher dose (Fig. 4A, **right panel**), suggesting a bell-shape rather than linear correlation between C16:0-Cer levels and VEGF-mediated vasodilation.

Only at high dose C24:0-Cer and C24:1-Cer were able to restore the vasorelaxation in response to VEGF (Fig. 4B and C). The Emax of VEGF concentration-response curve clearly shows the prevailing effects of C16:0-Cer vs. C24:0- and C24:1-Cer (Fig. 4D) also depicted in the schematic cartoon (Fig. 4E).

C16:0-Cer was also the most efficient in restoring S1P-mediated vasodilation compared to C24:0-Cer and C24:1-Cer (Fig. S3A-E). C16:0-Cer treatments restored also sphingomyelin levels in ECKO-Sptlc2 MA compared to vehicle treated (Fig. S3F). Unfortunately, despite including different branches, ceramide levels of MA were undetectable.

These data reveal specific biological functions of different ceramide species in preserving endothelial cell signaling and vasodilation.

C24:0-Cer and C24:1-Cer, but not C16:0-Cer, repristinate flow-mediated vasodilation in ECKO-Sptlc2 MA.

Low dose of C24:0-Cer and C24:1-Cer fully restored flow-mediated vasodilation in ECKO-Sptlc2 MA compared to controls (Fig. 5A, C, **left panels**), while at higher dose also C16:0-

cer re-established vasorelaxation to flow (Fig. 5A-C, **right panels**). The impact of specific ceramides on flow-induced vasodilation is schematized in Fig. 5E. Membrane biophysical properties are important for a proper mechanotransduction of changes in shear stress[38]. Most likely ceramides with short acyl chain and/or double bond ceramide might preserve mechanotransduction properties of the endothelium by increasing membrane fluidity.

Exogenous C16:0-Cer restores endothelial VEGFR2 signaling in EC lacking SPTLC2.

Mechanistic studies *in vitro* demonstrated that both genetic and pharmacological disruption of SPT activity impairs VEGFR2 signaling and downstream Akt-eNOS activation in murine ECKO-Stplc2 EC (Fig. S4) and HUVEC (Fig. 6A-D). It is noteworthy to mention that concentrations and durations of myriocin treatments have been carefully titrated in HUVEC (Fig. S5).

Interestingly, C16:0-Cer was able to rescue VEGFR2 signaling and Akt-eNOS phosphorylation, abrogated by myriocin treatment (Fig. 6A-D). In support of molecular signaling, exogenous C16:0-Cer was able to restore endothelial ceramide levels after 3h (Fig. 6E,F), but not hexosylceramides or sphingomyelins (Suppl. Fig. 6).

These data demonstrate that the *de novo* biosynthesis is necessary to maintain endothelial sphingolipid homeostasis, and reveal a prevailing role of C16:0-Cer in preserving endothelial cell signaling.

DISCUSSION

Altered sphingolipid levels are linked to hypertension, coronary artery disease, and cardiovascular complication in obesity and type 2 diabetes[6, 39]. Multiple clinical studies showed that specific plasma ceramides ratios, including C24:0/C16:0 and C22:0/C16:0 ceramides, independently correlate with major adverse cardiovascular events in patients with and without coronary artery diseases [5, 20, 40-43]. Recently, the Mayo clinic included plasma ceramide measurements as routine to predict at risk-patients with coronary artery diseases[44]. However, our knowledge of the role of ceramides in the pathophysiology of blood vessels remains limited.

Recently, we discovered a novel regulatory mechanism of sphingolipid *de novo* biosynthesis in blood vessels. Nogo-B, a membrane protein of the endoplasmic reticulum, binds to and downregulates the activity of SPT[26]. In absence of systemic or endothelial Nogo, mice are protected from hypertension[26], inflammation [28], and pathological cardiac hypertrophy[27], mainly mediated by an increased endothelial-derived S1P and S1P1 autocrine signaling. These studies suggested that Nogo-B-dependent downregulation of SPT in the vasculature has pathological implications and raised intriguing questions. First, considering that the endothelium is "bathed" by sphingolipid-rich plasma, is the *de novo* production of sphingolipids necessary to maintain endothelial sphingolipid homeostasis? Does endothelial SPT downregulation promote vascular dysfunction? Are changes in plasma ceramide indicative of changes in vascular SL metabolism and functions? Which SL impact endothelial functions the most? Considering the growing evidence implicating SL, particularly ceramides, in CV diseases, these are fundamental questions to address.

Our data clearly demonstrated that endothelial *de novo* biosynthesis is an important source of both plasma S1P and ceramide, but not sphingomyelins. While the endothelium[45], in addition to red blood cells[46], has been recognized to export S1P into the plasma with the aid of specific transporters[47, 48], much less is known on the sources and mechanisms of export of other circulating sphingolipids, including ceramides. Like most cells, EC can release heterogeneous microparticles from plasma membranes into the extracellular space in response to cell activation, injury and/or apoptosis. Microparticles derived from activated endothelium present higher ceramide content[49]. Accordingly, we reported an upregulation of SPT activity and accumulation of ceramide into the media of TNF-α-activated EC[27]. Altogether, these data put forward the consideration that remodeling of plasma ceramide profile in patients, preceding major cardiovascular events[5], might reflect not only changes in plasma lipoprotein composition, but also changes in endothelial-derived ceramides and vascular disease state.

S1P is a potent activator of eNOS[50], mainly via S1P1[51]. Previously, we demonstrated a crucial role for endothelial-derived S1P and autocrine S1P-S1P1-eNOS signaling in blood flow and pressure regulation[51]. In ECKO-Sptlc2 mice endothelial-derived S1P was markedly diminished (~40%; Fig. 1F), while flow-mediated vasodilation was blunted (Fig. 2F), supporting a critical, but not exclusive, role for endothelial autocrine S1P-eNOS signaling in flow-regulated vascular tone. Indeed, changes in membrane biophysical properties might also be implicated in the suppression of flow-mediated vasodilation in ECKO-Sptlc2 MA.

The increase of SPT activity in EC lacking Nogo-B leads to an upregulation of S1P-S1P1-eNOS autocrine signaling and lowers BP. The downregulation of SPT activity in EC lacking Sptlc2 while triggers a general impairment of endothelial-mediated vasodilation also lowers BP. Apparently contradictory, these findings can be reconciled by considering that the genetic deletion of Nogo-B, a modulator of SPT, has a different impact on sphingolipid profile than the deletion of SPT. In the latter model, low levels of ceramides can trigger an increased activation of eNOS at baseline (Fig 2, Fig. S4), via decrease of PP2A activity, which contributes to lower systemic BP and compensates for the loss of endothelial S1P signaling. While the analysis of LF/HF ratios exclude an impact of endothelial-derived sphingolipids on sympathetic system, clearly, as BP is the result of the interplay between blood vessels, heart, kidneys and brain, other players other than blood vessels can contribute to the reset blood pressure to lower than normal value, although the loss of SPT is specific to EC.

Our data suggest an important role of ceramides as "keeper" of EC signaling, most likely by dictating optimal biophysical properties of the membranes and/or partitioning of the receptors in specialized membrane microdomains[13, 52].

Specifically, while C16:0-cer exerts a prevailing role in restoring GPCR-, RTK-mediated vasodilation, C24:0- and C24:1-Cer preserve flow-mediate vascular tone regulation, suggesting that acyl chain length confers specific biological roles to ceramides in governing endothelial cell functions.

Most importantly, our data clearly demonstrate that the activation of Akt-eNOS-cGMP axis was preserved in ECKO-Sptlc2 MA (Fig. 2A-C , Fig. S2C) suggesting that the decrease in endothelial sphingolipids, although heightened Akt/eNOS basal phosphorylation, did not compromise the signal transduction of Akt-eNOS pathway.

A very interesting study of Oh P. & Schnitzer J. reported that Gq proteins preferentially segregate to caveolae, probably via interaction with caveolin-1, whereas Gs and Gi proteins locate to non-caveolar lipid rafts[54]. Our findings demonstrate that endogenous sphingolipids regulate GPCR-mediated signaling, except for Gq-coupled receptors, suggesting a different impact or segregation of sphingolipids in distinct specialized membrane domains. However, we do not know whether changes in endogenous sphingolipids differentially affect the lipid composition of membrane microdomains, and/or the partition of receptors and therefore their sensitivity towards ligand activation. Additional studies are needed to address these questions.

The finding that sphingolipid levels can impact specific endothelial signaling, and therefore blood flow and pressure, might have multiple clinical implications. First, sphingolipid accrual in obesity-driven type 2 diabetes has been implicated in vascular dysfunction via ceramide-activated PP2A, which in turn downregulates Akt/eNOS signaling[21, 22]. Although ceramide accrual has been demonstrated in aorta of hypertensive rats[6], sphingolipid levels in EC during hypertension have yet to be measured. Our findings suggest that altered endothelial ceramides contribute to vascular dysfunction by impairing receptor-mediated signaling, other than disruption of the Akt/eNOS axis. Second, about 50% of prescribed drugs target GPCR. Different studies reported the important role of sphingolipid in modulating the function of different membrane proteins, including GPCR[55, 56]. Thus, our findings have additional clinical implications as imply that derangement in sphingolipid metabolism and levels might impact GPCR-mediated signaling, and the effects elicited by drugs targeting GPCR[56].

Downregulation of SL *de novo* production impairs VEGFR2-mediated signaling and vasodilation following VEGF stimulation. Our data also complement the findings of Mehra V. et al. showing that C2-Cer treatment reduced basal and VEGF-activated Akt-eNOS phosphorylation and NO production, although in this study VEGFR2 phosphorylation was not assessed[57].

Our data on VEGF-induced VEGFR2 phosphorylation demonstrate that ceramides preserve the initiation[58] of signal transduction (Fig. 6, Fig. S4, S5).

In conclusions, our study reveal important pathophysiological implications of vascular-derived sphingolipids: 1) the *de novo* biosynthesis is a necessary to maintain vascular sphingolipid homeostasis and 2) regulate vascular tone and BP, mainly by preserving endothelial cell signaling and resting NO production; 3) in addition to S1P, the endothelium is a major determinant of plasma ceramide profile, clinical predictor of major cardiovascular events, 4) circulating ceramides may also be a "raconteur" of the health/diseased state of the endothelium.

Either accrual or depletion of SL has pathological implications, suggesting that SL needs to be tightly maintained within a narrow physiological window to preserve the "health state". Our study not only identifies biological functions of specific ceramide species in endothelial cell signaling, but also paves the way to a better understanding as to how sphingolipid derangement could contribute to endothelial dysfunction seen in metabolic syndrome and other cardiovascular diseases.

PERSPECTIVES

In recent years the importance of ceramides in cardiovascular and metabolic diseases has gained much attention. Multiple clinical studies established plasma ceramide as independent predictor of cardiovascular events in at risk-patients with coronary artery diseases. While, both accrual and depletion of SL has pathological implications, suggesting the need of a tight regulation to preserve the "health state", the impact of sphingolipids, particularly ceramides, on vascular functions and BP remains poorly understood.

Our study revealed a critical role of sphingolipid *de novo* biosynthesis in preserving endothelial cell functions and BP. Specifically, endothelial-derived ceramides emerge as critical regulator of receptor-mediated signaling, mechanotransduction and eNOS to preserve vascular and BP homeostasis.

Clinical implications of our findings extend to pathologies where ceramide is altered, including cardiovascular and metabolic diseases. Our study not only identifies biological functions of specific ceramide species in endothelial cell signaling, but also paves the way to a better understanding as to how sphingolipid derangement, particularly ceramide, could contribute to endothelial dysfunction seen in metabolic syndrome and other cardiovascular diseases, and could impact the therapeutic approach to treat those conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard abbreviations and acronyms

Ach	Acetylcholine
BP	Blood Pressure
Cer	Ceramide
GPCR	G-protein Coupled Receptors

EC	Endothelial Cell
MA	Mesenteric Artery
My	Myriocin
NO	Nitric Oxide
PE	Phenylephrine
RTK	Tyrosine Kinase Receptor
SL	Sphingolipid
SM	Sphingomyelin
SNP	Sodium Nitroprussiate
SPT	Serine Palmitoyltransferase
VSMC	Vascular Smooth Muscle Cell

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NOVELTY AND SIGNIFICANCE

What is new?

- Impaired endothelial *de novo* sphingolipid biosynthesis is sufficient to trigger vascular dysfunction and lower blood pressure.

- Plasma ceramide profile, predictor of major cardiovascular events, is dictated by the endothelium and might be a "raconteur" of the endothelial health/disease state

- Among SL, ceramides are necessary to preserves specific receptor-mediated signals and mechanotransduction pathways

What is relevant?

Altered sphingolipid homeostasis is causative of vascular dysfunction. Targeting sphingolipid metabolism to restore homeostasis may provide a therapeutic approach for hypertension and other related cardiovascular conditions underlined by sphingolipid derangements
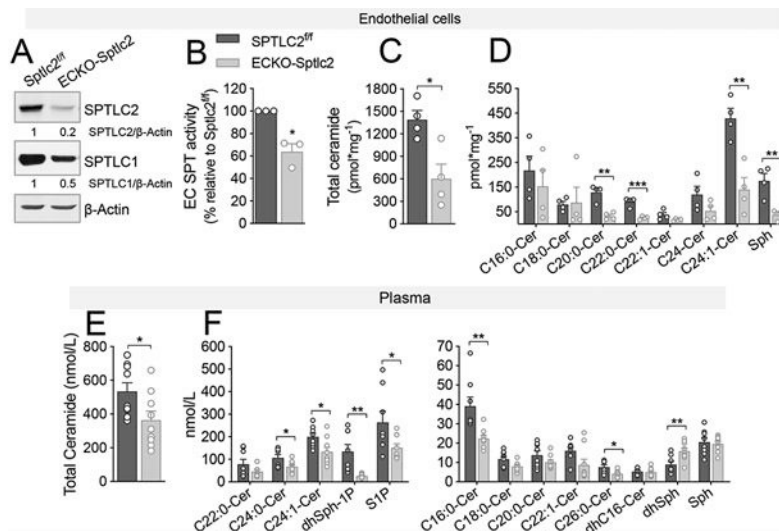
Figure 1. Endothelial sphingolipid *de novo* biosynthesis is an important source of local and circulating ceramide and S1P.

(**A**) WB and densitometric analysis of SPTLC1 and SPTLC2 in ECKO-Sptlc2 and Sptlc2^{f/f} EC after 4-OHT (1μM, 72h) treatment. β-actin, loading control. (**B**) SPT activity in ECKO-Sptlc2 and Sptlc2^{f/f} EC. [³H]-serine and palmitoyl-CoA were used as substrates for SPT. Sphinganine, the reaction product, was separated in TLC and quantified. (**C, D**) LC-MS/MS quantification of total and specific ceramides in Sptlc2^{f/f} and ECKO-Sptlc2 after 4-OHT treatment. (**A-D**) N 3 independent EC isolations/group; 4 mice/EC isolation. (**E, F**) LC-MS/MS analysis of ceramides, Sph and S1P in Sptlc2^{f/f} and ECKO-Sptlc2 plasma (n 9/group). dhSph-1P=Dihydro-sphingosine-1-phosphate, dhSph=dihydro-sphingosine, Sph=sphingosine. Data are expressed as mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to Sptlc2^{f/f}. Statistical significance was determined by Unpaired *t*-test.
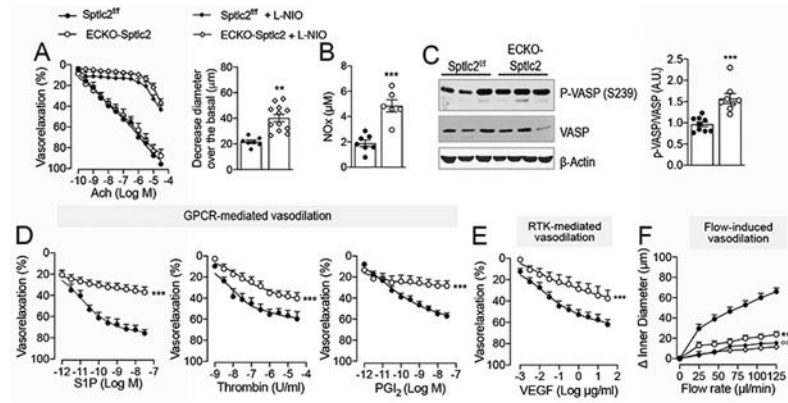
Figure 2. Endothelial-derived SL control vascular tone by preserving signaling transduction to agonists and flow.

(**A**) Ach-mediated vasodilation of MA in absence (n 12 mice/group, n 22 MA/group) and presence of L-NIO (100 μM, 20 min; n 5 mice/group n 8 MA/group), and L-NIO-induced vasoconstriction in Sptlc2$^{f/f}$ and ECKO-Sptlc2 MA at baseline (n 5 mice/group n 8 MA/group). (**B**) Plasma nitrite levels (n 6/group). (**C**) WB analysis for P-VASP and VASP on ECKO-Sptlc2 and Sptlc2$^{f/f}$ thoracic aortas (n=9 mice/group) and relative quantification. Vasodilation in response to: (**D**) Sphingosine-1-phosphate (S1P; n=9 mice/ group, n=9 MA/ group); thrombin (n 3 mice/group, n 4 MA/group); prostacyclin (PGI$_2$; n 4 mice/group, n 6 MA/group); (**E**) VEGF (n 5 mice/group, n 5 MA/group) and (**F**) flow (n 12 mice/ group, n 15 MA/group). All data represent mean ± SEM. ** p 0.01 and *** p 0.001 ECKO-Sptlc2 vs. Sptlc2$^{f/f}$. Statistical significance was determined by Unpaired t-test (**A, B, C**) or Two-way ANOVA (**A, D-F**).
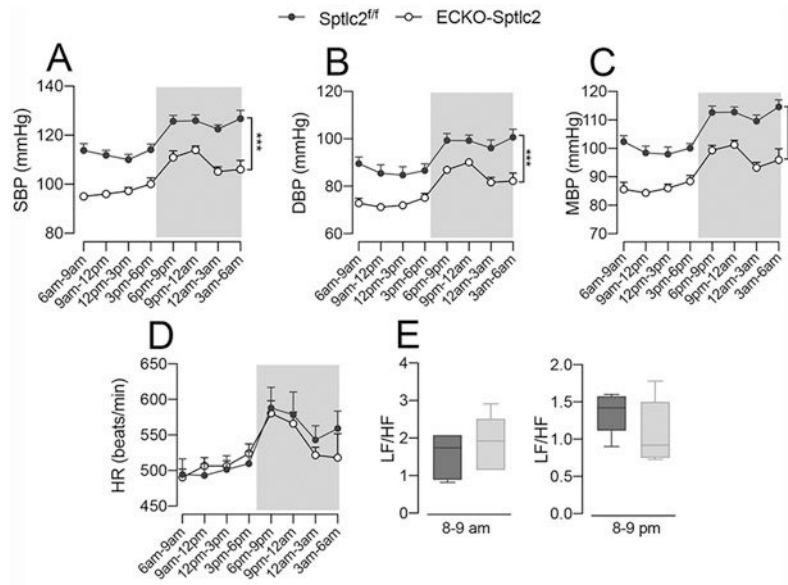
Figure 3. Radiotelemetry measurements of BP.
(**A**) Systolic, (**B**) diastolic and (**C**) mean BP, and (**D**) heart rate (HR) were measured by radiotelemetry for three consecutive days following the recovery from surgery (9 days) in ECKO-Sptlc2 (n=6) and Sptlc2$^{f/f}$ (n=6). (**E**) Analysis of low to high frequency (LF/HF) ratios in the same groups of mice. All data represent mean ± SEM. *** p 0.001 ECKO-Sptlc2 vs. Sptlc2$^{f/f}$ mice. Statistical analysis was performed with Two-way ANOVA with Sidak post-test (**A-D**) and unpaired t-test (**E**).
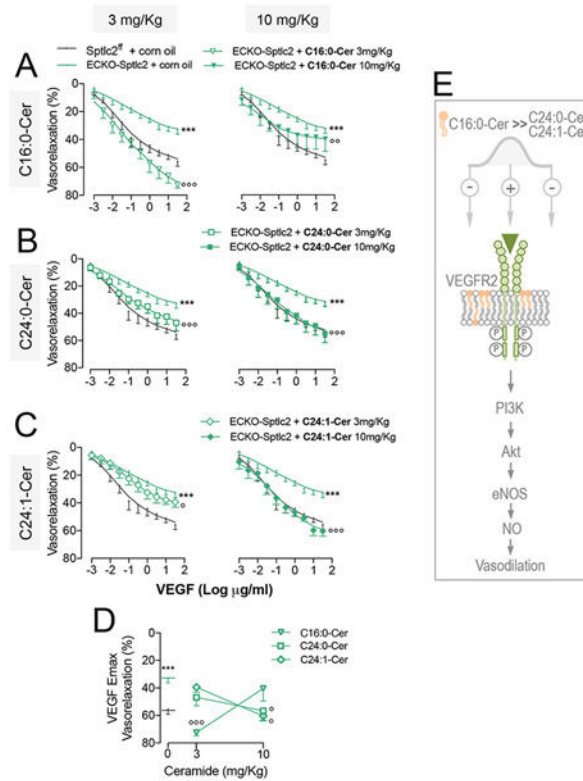
Figure 4. Prevailing role of C16:0-Cer vs. C24:0- and C24:1-Cer in restoring VEGF-mediated vasodilation.

ECKO-Sptlc2 mice were treated with C16:0-Cer, C24:0-Cer, C24:1-Cer at the doses of 3 mg/Kg/d (left panels) or 10 mg/Kg/d (right panels) i.p. for 2 consecutive days. VEGF-induced vasodilation in MA from ECKO-Sptlc2 mice treated with (**A**) C16:0-Cer (n ≥ 4 mice/group); (**B**) C24:0-Cer (n ≥ 4mice/group); (**C**) C24:1-Cer (n ≥ 4mice/group), or vehicle. (**D**) Maximum VEGF-induced vasodilation (Emax) of MA from ECKO-Sptlc2 mice treated with two different doses of C16:0-Cer, C24:0-Cer or C24:1-Cer compared to vehicle (indicated as 0 on X-axis). (**E**) Schematic carton representing the different ceramide-specific effects on VEGF-induced vasodilation. All data represent mean ± SEM. *** p ≤ 0.001 ECKO-Sptlc2 + corn oil vs. Sptlc2$^{f/f}$ + corn oil; ° p ≤ 0.05, °° p ≤ 0.01 and °°° p ≤ 0.001 ECKO-Sptlc2 + ceramide vs. ECKO-Sptlc2 + corn oil. Statistical significance was determined by Two-way ANOVA (**A,B,C**) or One-way ANOVA (**D**).
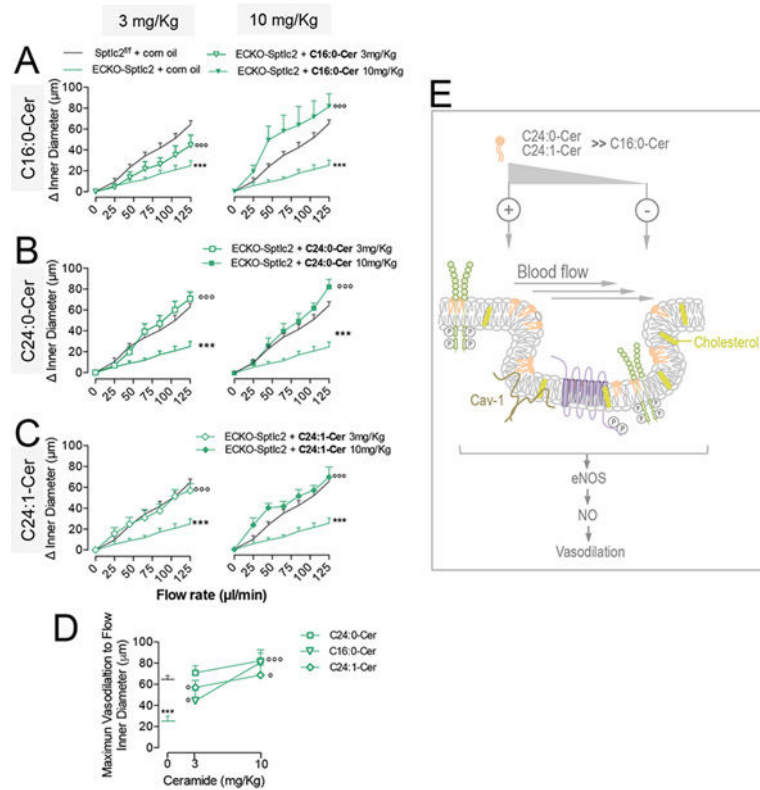
Figure 5. C24:0- and C24:1-Cer prevail over C16:0-Cer in restoring flow-mediated vasodilation in ECKO-Sptlc2 MA.

ECKO-Sptlc2 mice treated with C16:0-Cer, C24:0-Cer, C24:1-Cer at the doses of 3 mg/Kg/d (left panels) or 10 mg/Kg/d (right panels) i.p. for 2 consecutive days. Flow-induced vasodilation in MA from ECKO-Sptlc2 mice treated with (**A**) C16:0-Cer (n 4mice/group); (**B**) C24:0-Cer (n 4 mice/group); (**C**) C24:1-Cer (n 4 mice/group). (**D**) Flow-induced maximum vasodilation (Emax) of MA from ECKO-Sptlc2 mice treated with two different doses of C16:0-Cer, C24:0-Cer or C24:1-Cer compared to corn oil as vehicle. (**E**) Scheme representing specific effects of different ceramides on flow-induced vasodilation. Data are expressed as mean ± SEM. *** p 0.001 ECKO-Sptlc2 + corn oil vs. Sptlc2$^{f/f}$ + corn oil; ° p 0.05 and °°° p 0.001 ECKO-Sptlc2 + ceramide vs. ECKO-Sptlc2 + corn oil. Statistical significance was determined by Two-way ANOVA (**A, B, C**) or One-way ANOVA (**D**).
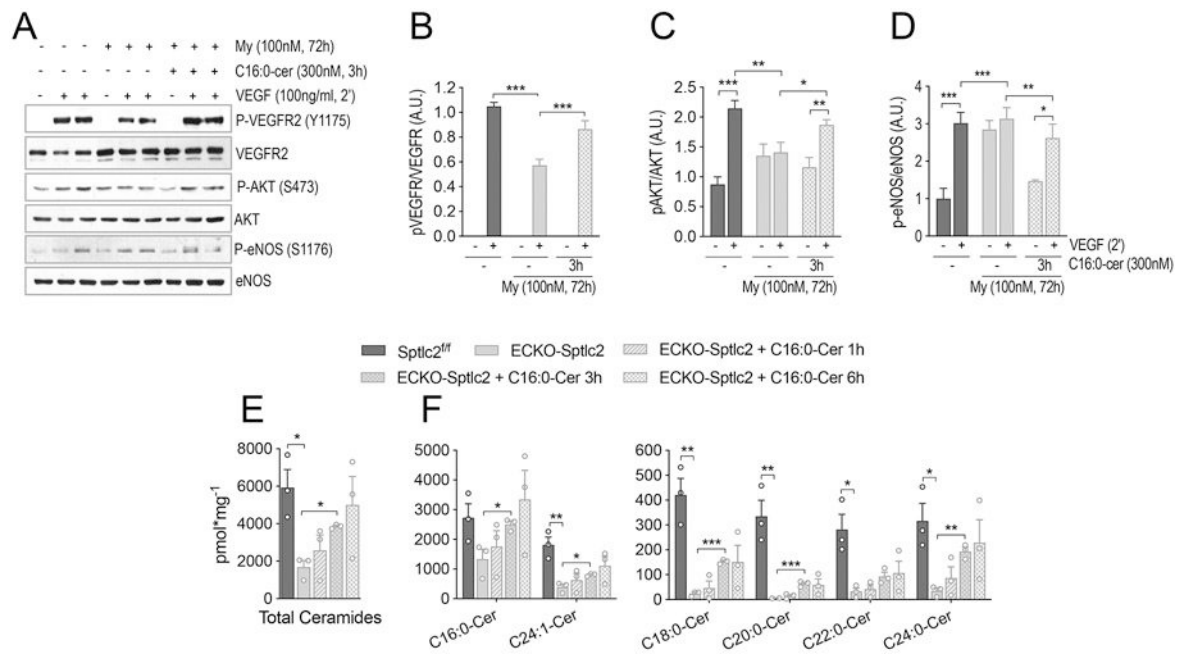
Figure 6. C16:0-cer restores VEGFR2-mediated signaling in EC treated with myriocin.
(**A**) WB analysis was performed on HUVEC treated with myriocin, inhibitor of SPT, or vehicle, followed by VEGF stimulation (100 ng/ml, 2 min). Some HUVEC treated with myriocin, were incubated with C16:0-Cer to restore VEGF signaling. Membranes were incubated with antibodies against P-VEGFR2 (Y1175), VEGFR2, P-AKT (S473), AKT, P-eNOS (S1176) and eNOS. (**B-C**) Densitometric analysis of indicated phospho/total protein ratios (n=4 independent experiments). (**E,F**) Sphingolipid measurements by LC-MS/MS in Sptlc2$^{f/f}$ and ECKO-Sptlc2 EC, treated with 300nM C16-Cer for the indicated times. (**E**) Total and (**F**) individual ceramides (3 independent EC isolations/group; 3 mice/EC isolation). Data are expressed as mean ± SEM. * p 0.05; ** *p* 0.01; *** p 0.001. Statistical significance was determined by One-way ANOVA.