ONLINE SUPPLEMENT

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

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SHORT TITLE
Ceramides control of endothelial cell functions

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MATERIALS AND METHODS

Vascular reactivity studies. At 2 weeks post-tamoxifen treatment, second order mesenteric arteries (MA) were harvested, cleaned from adhering tissue and mounted on glass micropipettes in a pressure myograph chamber (Danish MyoTechnology, Aarhus, Denmark), as previously described[1, 2]. Vessels were maintained in Krebs solution[1, 2]. MA were equilibrated for 15 min at 80 mmHg, pre-constricted with PE (1μM) and a cumulative concentration-response curve of Ach (0.1nM-30μM) was performed to evaluate the endothelial function. The following concentration-response curves were performed: histamine (10nM-30 μM), thrombin (1μU/ml-30mU/ml), sphingosine-1-phosphate (S1P, 1pM-30nM), insulin (1 pU/ml-3μU/ml) and VEGF (1μg/ml-30mg/ml), PE (1nM-30μM), U46619, TXA_2 analogue (1pM-3μM), isoproterenol (10nM-30μM), and sodium nitroprussiate (SNP, 10nM-30μM). Flow mediated vasodilation was evaluated as previously described[1].
In another set of experiments, MA were incubated with 10μM of N5-(1-Iminoethyl)-L-ornithine dihydrochloride (L-NIO), a non-selective NOS inhibitor, followed by cumulative concentration-response curve to Ach or stepwise increase of the flow.

Radiotelemetry BP measurements. SBP was measured in conscious $Sptlc2^{f/f}$ and ECKO-Sptlc2 male mice at 2-3 weeks post-tamoxifen treatment using Data Sciences International (DSI) implantable radiotelemetry transmitters[3]. Anesthetized (isoflurane, to effect) mice were implanted with carotid artery catheters advanced to the aortic arch and radiotelemeter implants (model HD-X10) inserted in a subcutaneous pocket on the back. After 9 days of recovery, BP was monitored continuously, with values reported every 5 s, for three consecutive days.

Low and high frequency ratio (LF/HF) between LF (0.40-1.5 Hz) domain and high frequency domain (1.5-4.0 Hz) were analyzed from 1h continuous telemetric BP record made between 8-9am and 8-9pm in undisturbed telemetered animals, by using Ponemah 6.x software. At study endpoint, mice were anesthetized with ketamine/xylazine (150/15 mg/kg, i.p.) for intracardiac perfusion with PBS and tissue harvest.

Sphingolipid measurements of MA. Following C16:0-ceramide (3mg/Kg/d for 2 days) or vehicle (corn oil) treatment, ECKO-Sptlc2 and Sptlc2f/f mice were sacrificed and a number of mesenteric arteries equal or more than 5 per mouse were dissected and cleaned from surrounding fat tissues, snap frozen on dry ice and stored at -80°C. SL were measured by the Collaborative Mass Spectrometry Core, Michigan State University.

Western Blot analysis for thoracic aortas. Dissected and fat-cleaned aortas were snap-frozen and immediately stored at -80 °C. After homogenization in RIPA buffer, aorta lysates were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as previously reported[1]. The following primary antibodies were used for WB analysis: phospho-S^{239}-VAPS and VASP (#3114 and #3132, Cell Signaling Technology), nNOS (#BML-SA227-0100, Enzo Life Sciences), iNOS (#15323, Abcam), β-actin (#A2228, Sigma).

Isolation of mouse liver and spleen EC. Liver and spleen EC, but not lung and heart EC, are responsive to 4-hydroxytamoxyfen-induced gene excision *in vitro*. Four-week-old female and male mice were used to isolate EC. Briefly, organs were cut into small pieces and incubated in a collagenase A (2mg/mL) and dispase (1U/mL) solution, followed by mechanical dissociation. EC were isolated with CD144 antibody-conjugated dynabeads. Isolated EC were cultured in DMEM with 20% FBS, 100 µg/mL heparin, and 25 µg/mL ECGF (Alfa Aesar, #J64516). For Sptlc2 gene excision, EC were treated with 4-hydroxytamoxyfen (1µM, 3 days). Before stimulation with VEGF (100ng/mL) or treatment with C16:0-cer (300nM), ECs were cultured in DMEM with 10% Charcoal-Stripped FBS for 18h, followed by 6h starvation in DMEM with 0.1% Charcoal-Stripped FBS.

Experimental protocol with HUVEC. HUVEC (LifeLine Cell Technology, cat# FC-0044) were grown in EBM-2 (Lonza, cat# CC-3156) supplemented with EGM-2 Endothelial Cell Growth Medium-2 BulletKit (Lonza, cat# CC-3162) with 10% FBS. Myriocin treatments were performed in EMB-2 (Lonza, cat# CC-3156) with 10% charcoal-stripped FBS at the indicated concentrations and times. Before stimulation with VEGF (100ng/mL, 2 min), cells were serum-starved for 6h in EBM-2 media. C16:0-Cer (300nM) or vehicle (ethanol, 1µL/mL) incubation was performed in the last 3h of myriocin incubation.

Western Blot analysis. RIPA buffer cell lysates were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as previously reported[1]. The following primary antibodies were used for WB analysis: phospho-Y^{1175}-VEGFR2, VEGFR2, phospho-S^{1177}-eNOS, phospho-S^{473}-AKT, and AKT (#2478, #2479, #9571, #4058, and #2920 respectively, Cell Signaling Technology); eNOS (#610297, BD Biosciences); Sptlc1 (#611305, BD Biosciences); Sptlc2 (A11716, ABclonal); β-actin (#A2228, Sigma).

Real-time PCR (RT-PCR) analysis of aortic EC and vascular smooth muscle cells (VSMC). EC and VSMC mRNA were isolated from $Sptlc2^{f/f}$ and ECKO-Sptlc2 aortas as previously described [1]. Briefly, dissected and fat-cleaned aortas were perfused with

TRIzol (Thermo Scientific) to collect EC lysates. The remaining aortas were homogenized in TRIzol. RT-PCR were performed with SYBR green PCR Master Mix (Qiagen, USA) by using iCycler Applied Biosystems 7700. The following primers were used: Sptlc2, forward (GGTCAGCCTCGGACAAGA) and reverse (TTGGCTCAGAAAGGCCAC); VE-cadherin, forward (TAGCAAGAGTGCGCTGGAGATTCA) and reverse (ACACATCATAGCTGGTGGTGTCCA); αSMA, forward (CAGGGAGTAATGGTTGGAAT) and reverse (TCTCAAACATAATCTGGGTCA); GAPDH, forward (AGGTCGGTGTGAACGGATTTG) and reverse (TGTAGACCATGTAGTTGAGGTCA).

SPT activity assay. SPT activity in EC was measured as previously described[1]. Briefly, the assay was conducted in a volume of 0.1 ml composed by: 0.1 M HEPES (pH 8.3 at 25 °C), 5 mM DTT, 2.5 mM EDTA (pH 7.4), 50 μM pyridoxal 5′-phosphate (PLP; Sigma), 0.45 μM [^{3}H]serine (PerkinElmer), 0.2 mM palmitoyl-CoA (Sigma) and 150 μg of protein lysates. After 15 min at 37 °C, the reaction was stopped with NH_4OH and the product 3-ketosphinganine converted into sphinganine with $NaBH_4$ (5 mg/ml). Radiolabeled lipids were extracted by using a modified Bligh and Dyer's method[1], dissolved in $CHCl_3$ and analyzed by thin-layer chromatography.

Plasma SL and nitrite measurements. Male mice at 2 weeks post-tamoxifen treatment were anesthetized with ketamine and xylazine (150/15 mg/kg). Whole blood was collected in EDTA and plasma obtained by centrifugation (1,000x *g*, 15 min, 4 °C) and immediately stored at -80 °C to measure SL and NO content[1]. SL were measured by the Lipidomics Analytical Core at the Medical University of South Carolina. Plasma NO levels were measured as nitrite (NOx) using a modified Griess reaction[1], as previously reported[1, 2].

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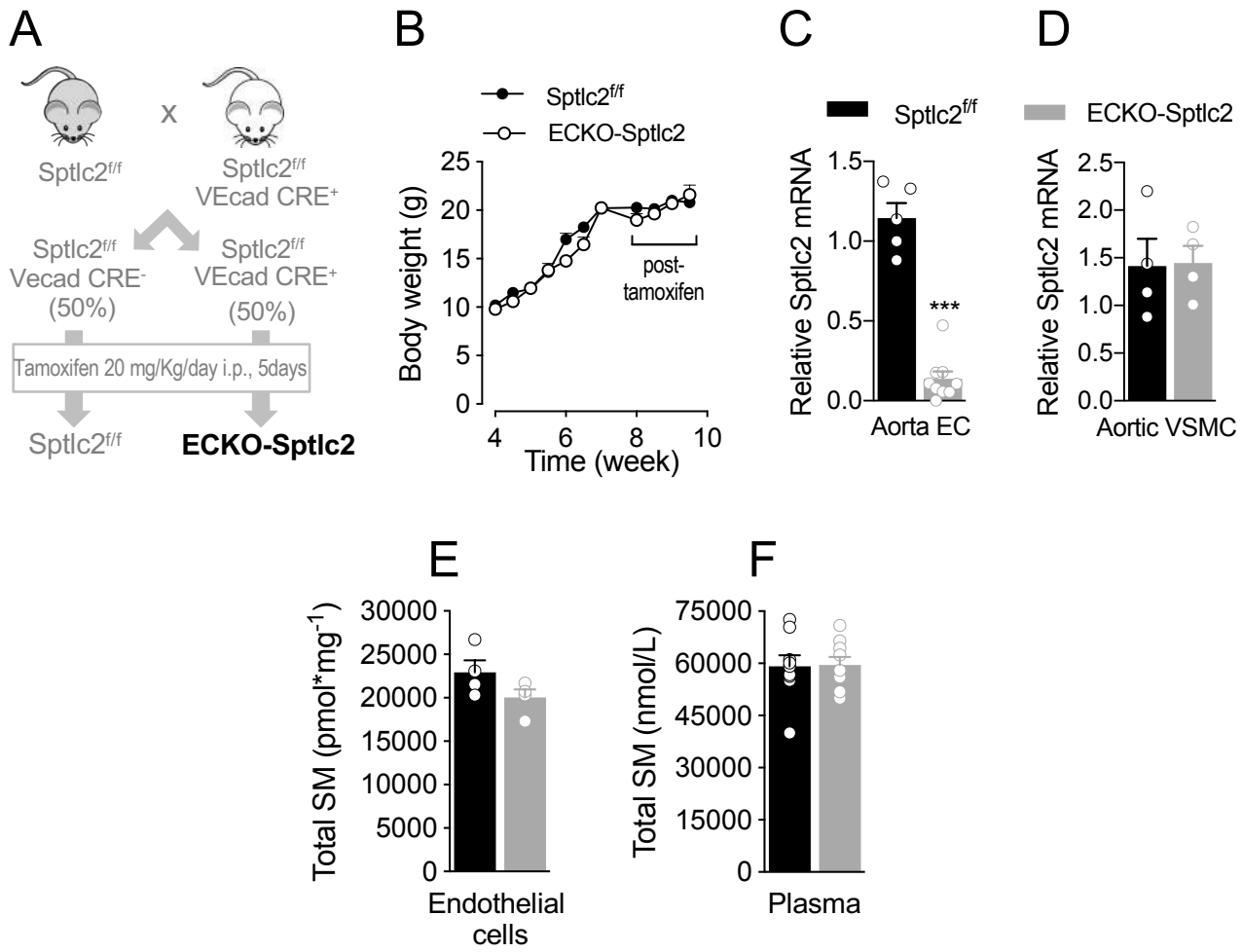

Figure S1. Generation and characterization of ECKO-Sptlc2 mice. (**A**) To obtain ECKO-Sptlc2 mice, Sptlc2$^{f/f}$ were crossed with Sptlc2$^{f/f}$ VE-Cad-CreERT2 (namely ECKO-Sptlc2) mice and obtained 50% of both genotypes as predicted by the Mendelian ratio. Seven weeks old mice were treated with tamoxifen (6 mg/Kg, i.p. for 5 consecutive days) to induce CRE-mediated *Sptlc2* excision. (**B**) Growth-curve of Sptlc2$^{f/f}$ and ECKO-Sptlc2 mice before and after tamoxifen treatment (Sptlc2$^{f/f}$ n≥6; ECKO-Sptlc2 n≥6). RT-PCR of aortic Sptlc2$^{f/f}$ and ECKO-Sptlc2 (**C**) EC (Sptlc2$^{f/f}$ n= 5; ECKO-Sptlc2 n=14) and (**D**) VSMC (Sptlc2$^{f/f}$ n=4; ECKO-Sptlc2 n=4). GAPDH was used as housekeeping gene. LC-MS/MS analysis of SM in (**E**) ECKO-Sptlc2 and Sptlc2$^{f/f}$ EC after 4-OHT treatments (4 independent EC isolations per group; 4 mice per EC isolation); and in (**F**) Sptlc2$^{f/f}$ and ECKO-Sptlc2 plasma (n≥ 9/group).

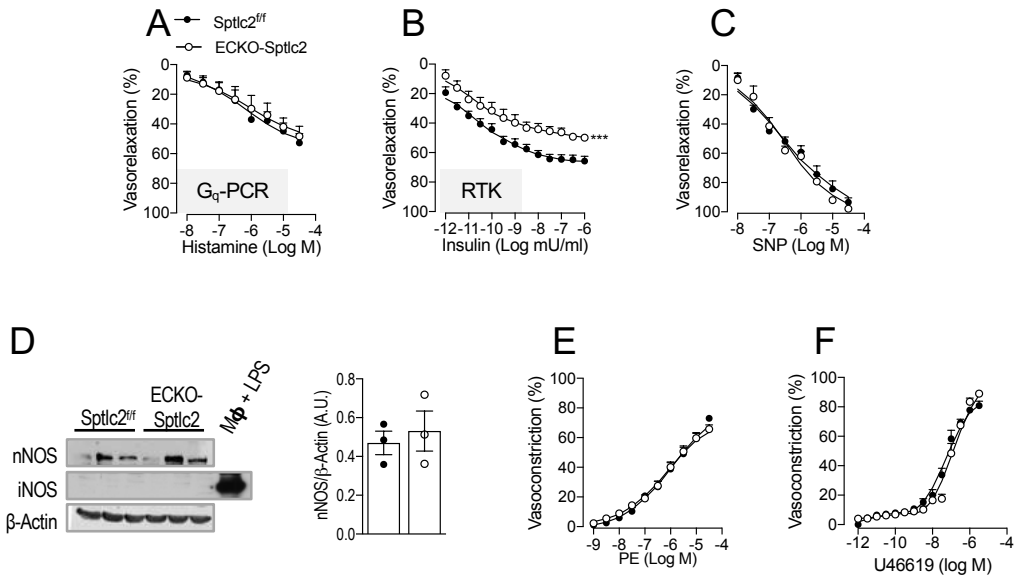

Figure S2. Endothelial sphingolipid *de novo* biosynthesis in vascular tone regulation. Vasodilation in response to: (**A**) Histamine (n≥3 mice/group, n≥4 MA/group); (**B**) insulin (n≥4 mice/group, n=8 MA/group); (**C**) Sodium nitroprussiate (SNP; n=4 mice/group, n≥5 MA/group) in Sptlc2$^{f/f}$ and ECKO-Sptlc2 MA. (**D**) WB analysis for nNOS and iNOS on ECKO-Sptlc2 and Sptlc2$^{f/f}$ thoracic aortas (n3≥mice/group). Vasoconstriction in response (**E**) phenylephrine (PE) (n≥13 mice/group, n≥22 MA/group); (**F**) U46619,TXA_2 analogue (n≥4 mice/group n≥5 MA/group). Data are expressed as the mean ± SEM. Statistical significance was determined by Two–way ANOVA.

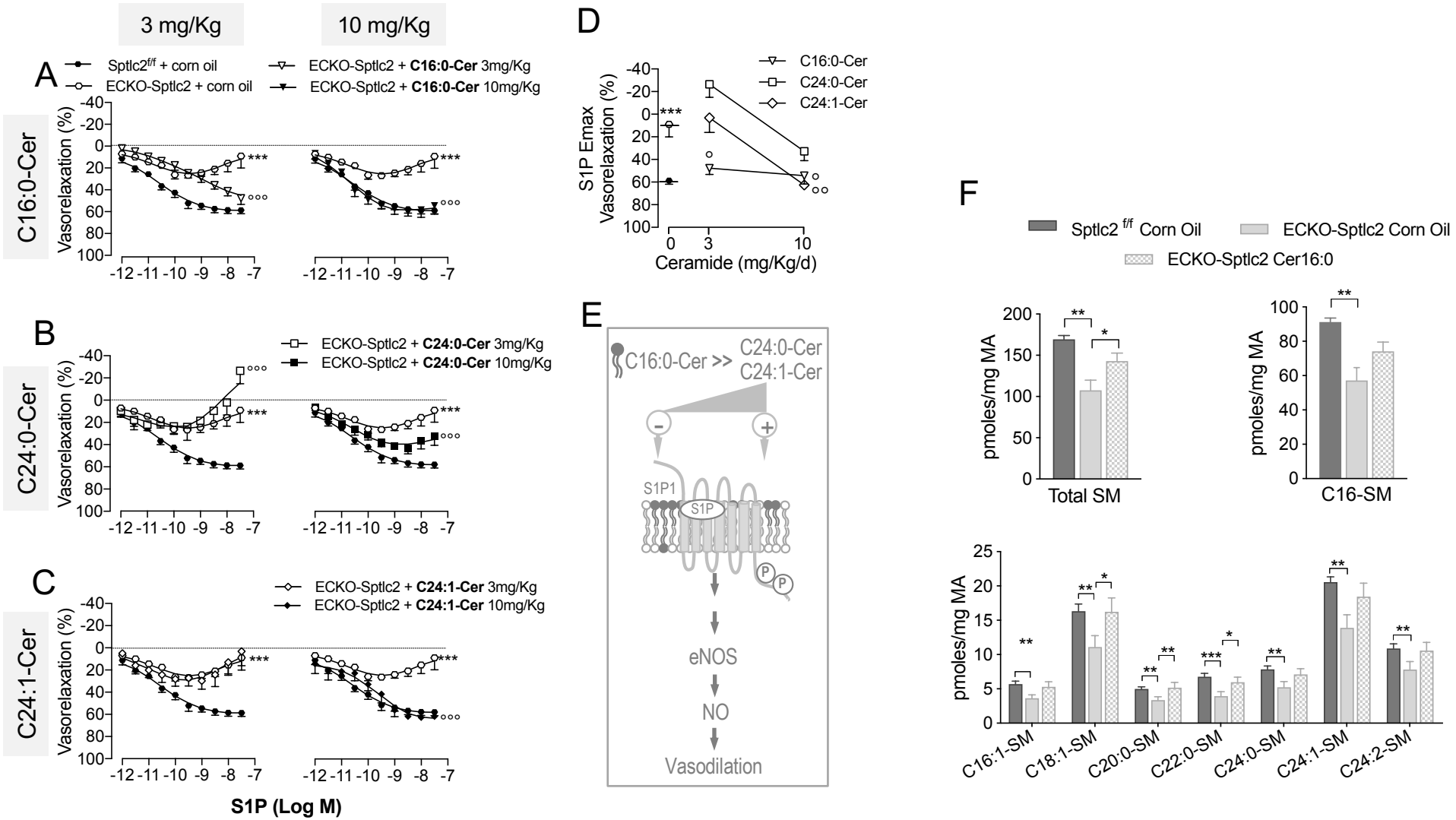

Figure S3. C16:0-Cer rescues S1P-mediated vasodilation and sphingomyelin levels in ECKO-Sptlc2 MA. S1P-induced vasodilation in ECKO-Sptlc2 mice treated with (**A**) C16:0-Cer (n≥ 4mice/group); (**B**) C24:0-Cer (n≥4 mice/group); (**C**) C24:1-Cer (n≥4 mice/group) at the doses of 3 mg/Kg/d (left panels) or 10 mg/Kg/d (right panels) i.p. for 2 consecutive days. (**D**) Maximum S1P-induced vasodilation (Emax) of MA from ECKO-Sptlc2 mice treated with different doses of C16:0-Cer, C24:0-Cer or C24:1-Cer. (**E**) Schematic representation of ceramide-specific effects on S1P-mediated vasodilation. (**F**) Total and specific SM measurements in MA (≥5 branches/MA) isolated from ECKO-Sptlc2 and Sptlc2$^{f/f}$ mice treated with corn oil, and ECKO-Sptlc2 mice treated with C16:0-Cer (3mg/Kg/d, for 2 days; n≥4mice/group). Data are expressed as mean ± SEM. For panels (**A-D**) *** 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. For panels (**F**) *p≤0.05, **p≤0.01.Statistical significance was determined by Two-way ANOVA (**A, B, C**) or One-way ANOVA (**D, F**).

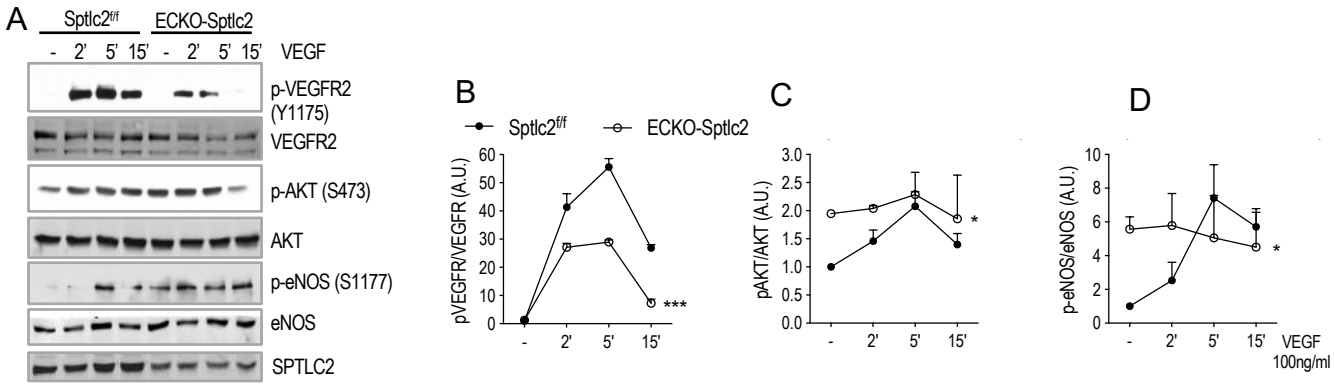

Figure S4. Endothelial VEGFR2 activation and downstream signaling is impaired in absence of SPT. (**A**) Primary Sptlc2$^{f/f}$ and ECKO-Sptlc2 EC were stimulated with VEGF (100 ng/ml) at different time points. WB analysis of P-VEGF (Y1175), VEGFR2, P-AKT (S473), AKT, P-eNOS (S1176) and eNOS (3 independent EC isolations/group; 4 mice/EC isolation). Densitometric analysis of (**B**) P-VEGFR2/VEGFR2, (**C**) P-AKT/AKT, and (**D**) P-eNOS/eNOS. Data represent mean ± SEM. * p≤0.5; *** *p*≤0.001 ECKO-Sptlc2 vs. Sptlc2$^{f/f}$. Statistical significance was determined by Two-way ANOVA.

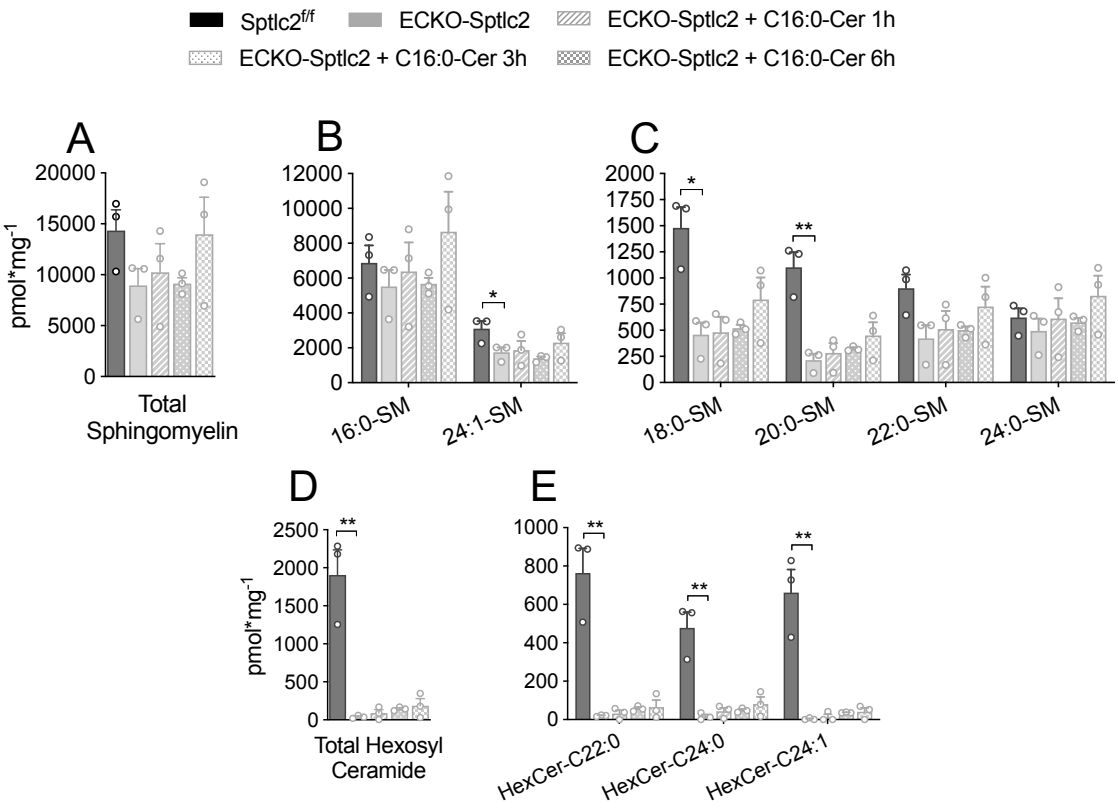

Figure S5. Sphingolipid measurements by LC-MS/MS in Sptlc2$^{f/f}$ and ECKO-Sptlc2 EC, treated with 300nM C16-Cer for the indicated times. (**A**) Total and (**B, C)** individual sphingomyelins; (D) total and (E) individual hexosylceramides (3 independent EC isolations/group; 3 mice/EC isolation). Data are expressed as mean ± SEM. * p≤0.05; ** *p*≤0.01; *** p≤0.001. Statistical significance was determined by One-way ANOVA.

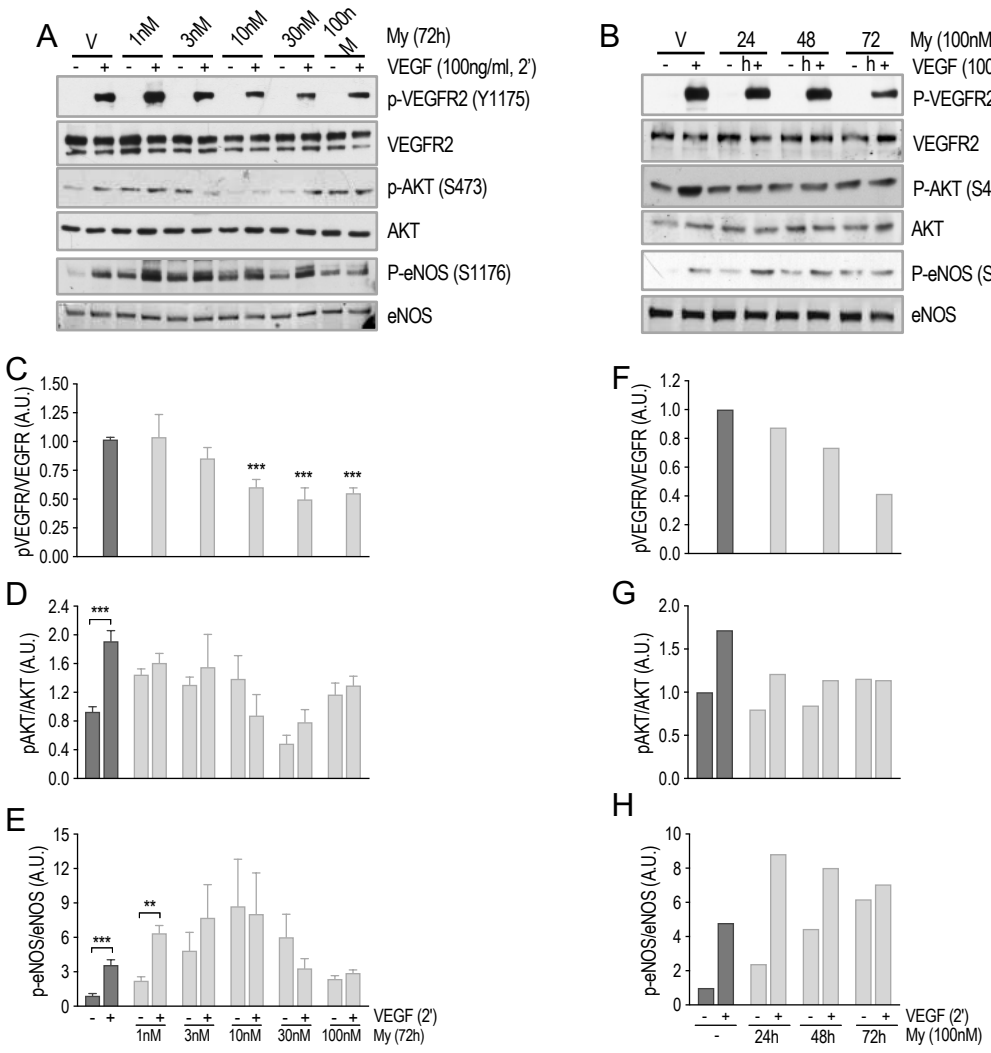

Figure S6. WB analysis was performed on HUVEC treated with myriocin, inhibitor of SPT, at different concentrations **(A)** or time **(B)** followed by VEGF stimulation (100 ng/ml, 2 min). Membranes were incubated with antibodies against P-VEGFR2 (Y1175), VEGFR2, P-AKT (S473), AKT, P-eNOS (S1176) and eNOS. (**B-H**) Densitometric analysis of indicated phospho/total protein ratios (n=4 independent experiments. Data are expressed as mean ± SEM. * p≤0.05; ** *p*≤0.01; *** p≤0.001. Statistical significance was determined by One-way ANOVA.