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ORIGINAL RESEARCH

Endothelial Spns2 and ApoM Regulation of Vascular Tone and Hypertension Via Sphingosine-1-Phosphate

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BACKGROUND: Most of the circulating sphingosine-1-phosphate (S1P) is bound to ApoM (apolipoprotein M) of high-density lipoprotein (HDL) and mediates many beneficial effects of HDL on the vasculature via G protein–coupled S1P receptors. HDL-bound S1P is decreased in atherosclerosis, myocardial infarction, and diabetes mellitus. In addition to being the target, the endothelium is a source of S1P, which is transported outside of the cells by Spinster-2, contributing to circulating S1P as well as to local signaling. Mice lacking endothelial S1P receptor 1 are hypertensive, suggesting a vasculoprotective role of S1P signaling. This study investigates the role of endothelial-derived S1P and ApoM-bound S1P in regulating vascular tone and blood pressure.

METHODS AND RESULTS: ApoM knockout (ApoM KO) mice and mice lacking endothelial Spinster-2 (ECKO-Spns2) were infused with angiotensin II for 28 days. Blood pressure, measured by telemetry and tail-cuff, was significantly increased in both ECKO-Spns2 and ApoM KO versus control mice, at baseline and following angiotensin II. Notably, ECKO-Spns2 presented an impaired vasodilation to flow and blood pressure dipping, which is clinically associated with increased risk for cardiovascular events. In hypertension, both groups presented reduced flow-mediated vasodilation and some degree of impairment in endothelial NO production, which was more evident in ECKO-Spns2. Increased hypertension in ECKO-Spns2 and ApoM KO mice correlated with worsened cardiac hypertrophy versus controls.

CONCLUSIONS: Our study identifies an important role for Spinster-2 and ApoM-HDL in blood pressure homeostasis via S1P-NO signaling and dissects the pathophysiological impact of endothelial-derived S1P and ApoM of HDL-bound S1P in hypertension and cardiac hypertrophy.

Key Words: apolipoprotein ■ high blood pressure ■ hypertension ■ vascular tone regulation

Sphingosine-1-phosphate (S1P), a potent bioactive sphingolipid, controls different physiological processes including immune cell trafficking and vascular development and homeostasis[1-3] via 5 G-protein–coupled S1P receptors, namely, S1P1-5.[4,5] S1P1-3 are expressed throughout the cardiovascular system,[6,7] with S1P1 being the most abundant in the endothelium.[8] The loss of S1P1 is embryonically lethal because of vascular defects,[9] underscoring the crucial role of S1P signaling in development. Postnatally, S1P signaling contributes to maintaining cardiovascular homeostasis by preserving the endothelial barrier function[8,10] and potently stimulating NO production by endothelial NO synthase (eNOS).[11,12] Recently, we demonstrated that endothelial S1P1-NO signaling is of critical importance in blood flow and pressure regulation.[13] Genetic ablation of endothelial S1P1 or chronic administration of fingolimod, a functional antagonist reducing S1P1 expression,[14,15] impaired flow-induced vasodilation of resistant arteries, and increased blood

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CLINICAL PERSPECTIVE

What Is New?

- Endothelial spinster-2 is necessary to preserve the vasodilation to flow, blood pressure homeostasis, and dipping pattern via S1P signaling.
- The endothelium is not responsible for plasma sphingosine-1- phosphate (S1P) increase in hypertension.
- Plasma S1P/C16:0-cer and C24:0/C16:0-cer ratios correlate with hypertension; the loss of both spinster 2 transporter and ApoM results in increased blood pressure, worsening hypertension, and cardiac hypertrophy.

What Are the Clinical Implications?

- S1P signaling might be a promising therapeutic target to lower blood pressure, re-establish the physiological dipping pattern, and protect the heart from hypertrophy.
- Plasma S1P/C16:0-cer and C24:0/C16:0-cer could serve as potential biomarkers for vascular diseases such as hypertension.

Nonstandard Abbreviations and Acronyms

AngII	angiotensin II
eNOS	endothelial nitric oxide synthase
L-NIO	N5-(1-Iminoethyl)-L-ornithine dihydrochloride
MA	mesenteric artery
S1P	sphingosine-1-phosphate
S1P1	sphingosine-1-phosphate receptor 1
Spns2	spinster 2 transporter

pressure (BP) in mice[13] and humans,[16] suggesting a protective role of S1P signaling on the vasculature.

In addition to red blood cells,[17] endothelial cells are an important source of plasma S1P,[18-20] which is secreted via a specific transporter, Spinster-2.[20] Systemic and endothelial deletion of *Spns2* significantly reduces plasma S1P, as well as lymph S1P, causing lymphopenia.[20,21] Once outside of the cells, S1P can signal to its receptors in an autocrine/paracrine manner or bind to circulating carriers.

We have previously demonstrated that systemic and endothelial loss of Nogo-B, an inhibitor of sphingolipid de novo biosynthesis, enhances endothelial-derived S1P and S1P1-NO signaling axis protecting the mice from hypertension, heart failure, and inflammation.[22-24] These data suggest that endothelial-derived S1P contributes to maintaining cardiovascular homeostasis.

ApoM of high-density lipoprotein (HDL) is the major carrier of S1P in the plasma (≈65%), with albumin and ApoA4 accounting for the remaining fraction.[25-27] Although the role of ApoM is not limited to S1P binding, S1P can interact with an amphiphilic pocket in the lipocalin fold of ApoM,[26] and can displace the myristic acid with an IC_{50} of 0.9 μmol/L.[28] A variety of studies demonstrated that S1P mediates many of the cardiovascular beneficial effects attributed to HDL, including the activation of eNOS.[29,30] Clinically, low levels of HDL-bound S1P strongly correlate with cardiovascular diseases, including atherosclerosis, coronary artery diseases, and myocardial infarction.[31-33] *APOM* single nucleotide polymorphism has been associated with atherosclerosis.[34,35] Mice lacking ApoM have reduced plasma S1P (≈50%), enhanced permeability, and inflammation,[26,36] underscoring the vasculoprotective role of circulating HDL-bound S1P.

Thus, the aim of our study was to investigate the role of endothelial-derived and ApoM-bound S1P in vascular tone and BP homeostasis by using mice knockout for *Spns2* and *Apom,* respectively. Our results showed that the loss of Spns-2 impaired flow-mediated vasodilation and BP dipping at baseline, whereas both ApoM and Spns-2 exacerbate hypertension and cardiac hypertrophy in mice infused with Ang-II. Mechanistically, a reduced S1P signaling impairs basal endothelial NO production and vasodilation to flow, leading to increase in vascular dysfunction and BP. Interestingly, plasma ceramide and S1P changes in hypertension, as well as in ECKO-Spns2 mice, resemble the ones reported for patients with coronary heart disease,[37,38] suggesting that impaired endothelial S1P signaling might contribute to the onset of and/or exacerbate vascular diseases.

METHODS

Mouse Models

The data that support the findings of this study are available from the corresponding author upon reasonable request. Animal experiments were conducted according to the protocols approved by the Weill Cornell Institutional Animal Care and Use Committee. All the studies were performed in male mice at the age of 12 to 16 weeks and the mice were fed a standard chow diet. Floxed-Spns-2 mice were crossed with VE-Cadherin-CRE-ER^{T213} to generate mice lacking Spns-2 specifically in endothelial cells, here thereafter referred to as ECKO-Spns2. Gene excision was achieved by intraperitoneal injection of tamoxifen (20 mg/kg per day; for 5 consecutive days) and experiments were performed at 3 weeks post-tamoxifen treatment. Spns2$^{f/f}$ were also treated with tamoxifen (20 mg/kg per day; for 5 days) and used as controls. ApoM knock out (KO)

mice have been backcrossed more than 8 times with C57BL/6J mice and previously described.[26] All mice were born with Mendelian frequencies.

Real-Time Polymerase Chain Reaction on Thoracic Aorta

Real-time polymerase chain reaction for spns2 expression was performed on ECKO-Spns2 and Spns2$^{f/f}$ thoracic aortas to evaluate the efficiency of excision. Total mRNA from tissue was extracted according to the TRIzol reagent protocol (Thermo Scientific). Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat# K1641) was used for the reverse transcription of 100 ng of RNA. For polymerase chain reaction analysis, SYBR green PCR Master Mix (Qiagen, USA) and iCycler Applied Biosystems 7700 were used. *Spns2* primers: forward (AGAAGCCGCATCCTCAGTTAGC), reverse (CAGGCCAGAATCTCCCCAAATC). *18S* primers: forward (TTCCGATAACGAACGAGACTCT), reverse (TGGCTGAACGCCACTTGTC). *Spns2* relative mRNA expression was calculated with the 2(–ΔΔ*C*t) method, using 18S as housekeeping.[39]

Chronic Infusion of Angiotensin-II

Hypertension was induced by chronic infusion of angiotensin-II (AngII) (500 ng/kg per minute) with an osmotic mini-pump (Alzet Model 2004) implanted subcutaneously in ECKO-Spns2, ApoM KO, and control littermate age-matched mice. Animals were randomly assigned to receive either AngII or vehicle-saline as controls. BP was measured before and once a week following chronic infusion of AngII for 28 days by using the tail-cuff system. At study end point, mice were anesthetized with ketamine/xylazine (150/15 mg/kg, ip), blood was collected with an intracardiac puncture, and tissues were harvested after PBS perfusion.

BP Measurements by Radiotelemetry and Tail Cuff

Systolic BP (SBP) was measured in conscious Spns2$^{f/f}$ and ECKO-Spns2 male mice (n=5/group) using Data Sciences International (DSI) implantable radiotelemetry transmitters.[40] Mice anesthetized with ketamine/xylazine (150/15 mg per kg) 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 seconds, for 3 consecutive days and expressed as 3-hour interval average.

Low- and high-frequency ratio between low-frequency (0.40–1.5 Hz) domain and high-frequency domain (1.5–4.0 Hz) were analyzed from 1 hour continuous telemetric BP record made between 10 AM and 12 PM and 10 PM and 12 AM in undisturbed telemetered animals, by using Ponemah 6.x software, as previously described.[19] BP dipping was calculated by subtracting the mean diurnal BP from the mean nocturnal BP.

SBP was also measured by the noninvasive tail-cuff method (Hatteras SC1000, Cary, NC) in conscious mice. Before BP measurements, mice were acclimatized for 1 hour and then placed in a chamber preheated at 34°C with a pulse sensor around their tails for BP recordings. Mice were trained for 5 to 9 consecutive days. Recording sessions consisted of 3 precycles (not used for the analysis) to accustom the animals, followed by 10 consecutive measurements for 3 consecutive days. All basal values collected were averaged. Following AngII infusion, SBP was recorded once per week for 4 weeks.

Wheat Germ Agglutinin Staining

Mouse hearts were fixed with 4% paraformaldehyde overnight at 4°C, divided into 3 parts (base, center, and apex), paraffin embedded, and cut into 4-µm sections. Myocardial sections were stained with 40 µg/mL wheat germ agglutinin (W7024; Invitrogen) in PBS for 1.5 hours at room temperature in order to label cardiomyocyte membranes. Nuclei were counterstained with 4'6-diamidino-2-phenylindole. Immunofluorescence images of heart sections were captured with Zeiss LSM Meta microscope and LSM Image Browser software (Carl Zeiss). Cross-sectional area was analyzed with ImageJ.

Sphingolipid Analysis by Liquid Chromatography–Tandem Mass Spectrometry

Spns2$^{f/f}$ and ECKO-Spns2 blood was collected via intracardiac puncture in EDTA to a final concentration of 50 mmol/L. Plasma was obtained by centrifugation (1000*g*, 15 minutes, 4°C) and immediately stored at –80°C for sphingolipid (SL) measurements via liquid chromatography–tandem mass spectrometry by the Lipidomics Analytical Core at the Medical University of South Carolina.

Western Blot

Western blot was performed as previously described.[19] Briefly, aortas from Spns2$^{f/f}$ and ECKO-Spns2, normotensive and hypertensive mice were harvested and cleaned from connective tissue and snap-frozen individually in liquid nitrogen. Each aorta had been homogenized in RIPA buffer and analyzed by Western blot. The following primary antibodies were used: S1P1 1:1000 ON (#S12935; Abclonal),

eNOS 1:2000 ON (#610297, BD bioscience), phospho-S239- vasodilator-stimulated phosphoprotein 1:1000 ON and vasodilator-stimulated phosphoprotein 1:1000 ON (#3114 and #3132, Cell Signaling Technology), and β-actin 1:5000 1h (#A2228; Sigma Aldrich, St. Louis, MO).

Vascular Reactivity Studies by Using the Pressure Myograph System

Second-order mesenteric arteries (MA) were cleaned from surrounding fat tissue and mounted on glass micropipettes by keeping the same orientation of the flow in a pressure myograph chamber (DanisMyoTechnology, Aarhus, Denmark) as previously described.[22] Briefly, vessels were perfused with oxygenated (95% O_2 and 5% CO_2) Krebs solution (mmol/L: NaCl 118, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10.1) at 37°C, and maintained at 80 mm Hg for 30 minutes, before preconstriction with phenylephrine (1×10^{-6} mol/L) followed by a cumulative concentration-response curve of acetylcholine (1×10^{-10} to 3×10^{-5} mol/L) to assess the integrity of the endothelium. Vessels with acetylcholine-induced vasodilation lower than 70% were excluded. Concentration-response curves of phenylephrine (1×10^{-9} to 3×10^{-5} mol/L) and S1P (1×10^{-12} to 3×10^{-9} mol/L) were performed. Flow-dependent vasodilation and myogenic tone were also assessed as previously described.[22] MA were incubated with 100 μmol/L of N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO, Tocris, 15 minutes), nonspecific inhibitor of eNOS[41] followed by vasodilation in response to acetylcholine to assess basal and stimulated NO production.

Statistical Analysis

Two-way ANOVA with Sidak's post-test, 2-way ANOVA with Tukey's post-test, or Student *t* test were used for the statistical analysis as indicated in figure legends. Differences were considered statistically significant when $P<0.05$. GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA) was used for all statistical analysis.

RESULTS

Endothelial S1P Transporter, Spns2, Is Necessary for BP Homeostasis and Dipping

Endothelial Spns-2 excision in thoracic aortas following tamoxifen treatment was >80% (Figure 1A). Interestingly, radiotelemetry measurements showed a significant increase in systolic, diastolic, and mean BP (Figure 1B through 1D) in ECKO-Spns2 versus Spns2$^{f/f}$ mice, particularly during daytime, with no difference in heart rate (Figure 1E). The higher BP in ECKO-Spns2 was not because of an increased sympathetic outflow because low frequency/high-frequency ratios during light and dark cycles were comparable (Figure 1F). However, night–day BP differences showed an impaired dipping phenotype in ECKO-Spns2 mice compared with Spns2$^{f/f}$ (Figure 1G). These data suggest an important role of endothelial-derived S1P in regulating circadian BP pattern and maintaining BP homeostasis.

Loss of Spns2 Exacerbates Hypertension and Cardiac Hypertrophy and Correlates With a Pathological Profile of Plasma Ceramide

In agreement with radiotelemetry data, the tail-cuff approach was able to measure similar differences in SBP between ECKO-Spns2 and Spns2$^{f/f}$ mice at baseline (120.8±2.1 versus 106.6±1.3 mm Hg; n=8). Thus, SBP measurements after AngII infusion were performed with the tail-cuff system. Interestingly, SBP in ECKO-Spns2 mice was significantly higher than in controls throughout the 28 days of chronic AngII infusion (156.0±1.2 versus 142.0±1.6 mm Hg, n=8; Figure 2A) and correlated with increased cardiac hypertrophy and cardiomyocyte area (Figure 2B and 2C), suggesting a protective role of endothelial-derived S1P in hypertension and pathological cardiac hypertrophy.

As reported in previous studies in mice and patients,[42,43] plasmatic levels of S1P were increased in hypertension (Figure 2D). Hence a pathological role for circulating S1P was postulated in this condition. Interestingly, plasma S1P is reduced in ECKO-Spns2 versus Spns2$^{f/f}$ (≈50%, Figure 2D), in agreement with previous studies,[20] and although increased in AngII-induced hypertension, S1P remained significantly lower than in Spns2$^{f/f}$ mice. Nonetheless, SBP of ECKO-Spns2 mice was significantly higher than in Spns2$^{f/f}$ mice, arguing against a pathological role of plasma S1P in hypertension but rather supporting a protective function. Furthermore, ECKO-Spns2 and Spns2$^{f/f}$ showed the same increase in plasma S1P in hypertension (Figure 2E), suggesting that other sources rather than the endothelium are accountable for the increase in circulating S1P in this condition.

Hypertension does not alter plasma sphingomyelin profile in mice (Figure S1). Although the overall ceramide profile did not change in hypertension (Figure S2), specific ceramide species changed (Figure 2F and 2G). Interestingly, similar to patients affected by coronary artery disease,[38,44,45] the C24:0-cer/C16:0-cer ratio was significantly decreased in hypertension (Figure 2H). We also found that S1P/C16:0-cer ratios rather than S1P levels are markedly decreased in hypertensive mice (Figure 2I). Strikingly, the loss of endothelial Spns2 results in a pathological C24:0-cer/C16:0-cer and S1P/

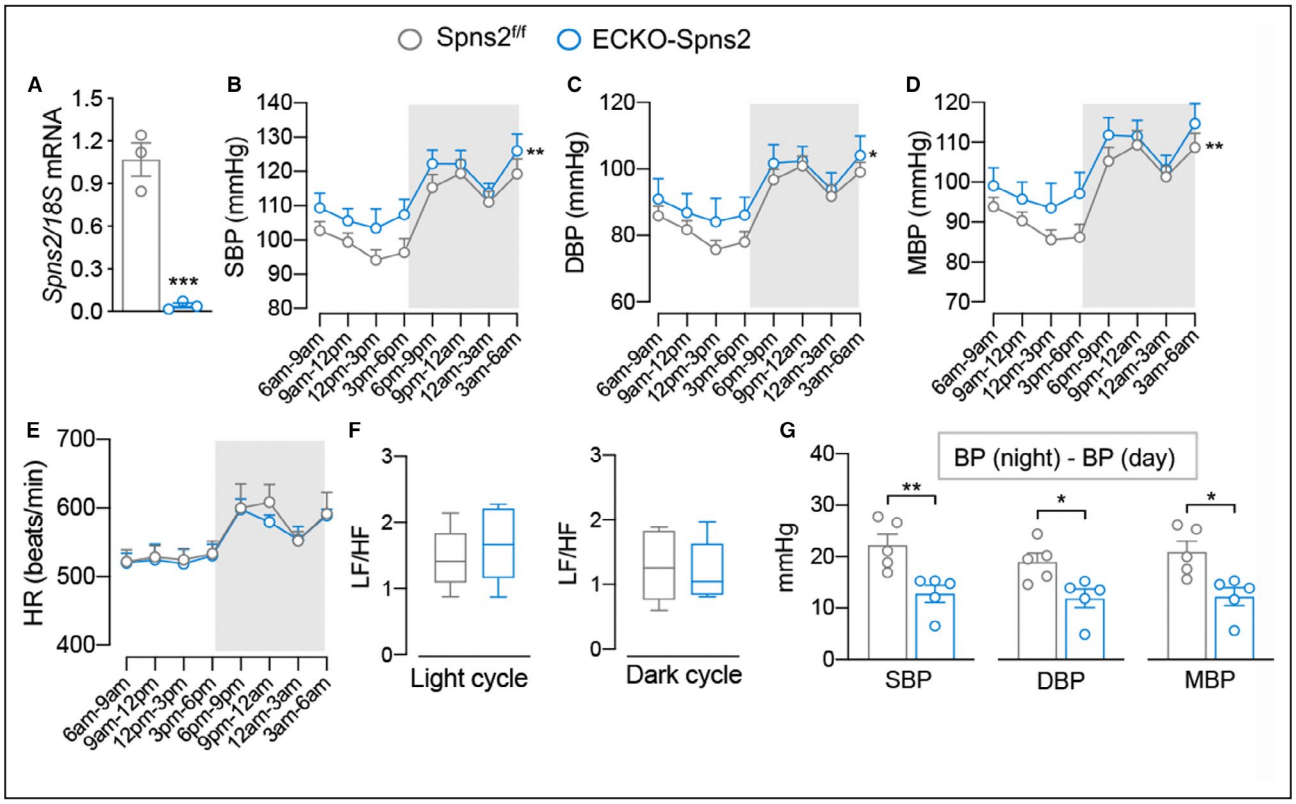

Figure 1. Endothelial S1P transporter, *Spns2*, is necessary for BP homeostasis and dipping.
A, RT-PCR of aortic Spns2$^{f/f}$ and ECKO-Spns2 mice. Radiotelemetry measurements of (**B**) SBP, (**C**) DBP, (**D**) MBP, and (**E**) heart rate (HR) in ECKO-Spns2 (n=5) and Spns2$^{f/f}$ (n=5) mice. **F**, Analysis of low/high frequency (LF/HF) ratios. **G**, Differences of BP between dark and light cycle in the same groups of mice (n=5 mice/group). Data are expressed as mean±SEM. *P≤0.05; **P≤0.01; ***P≤0.01. Statistical significance was determined by unpaired *t* test (**A**, **F**, and **G**) or 2-way ANOVA with Sidak's post-test (**B** through **E**). BP indicates blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; RT-PCR, real-time polymerase chain reaction; S1P, sphingosine-1-phosphate; and SBP, systolic blood pressure.

C16:0-cer ratio that correlates with higher BP, suggesting that endothelial-derived S1P is critical to preserve BP homeostasis and circulating S1P and ceramide profile within a physiological range.

Spinster-2 Is Necessary for Flow-Mediated Vasodilation Via Local S1P-NO Signaling

We reported that endothelial S1P1 signaling is critical in flow-induced vasodilation.[13] Interestingly, flow-mediated vasodilation was significantly reduced in MA of ECKO-Spns2 at baseline and in AngII-induced hypertension (Figure 3A), supporting the direct role of endothelial-derived S1P in mechanotransduction signaling in response to flow via S1P1. The reduction in vessel diameter following L-NIO, a nonspecific inhibitor of eNOS, is an indirect index of eNOS-derived NO production. L-NIO-induced vasoconstriction was significantly reduced in ECKO-Spns2 MA in vehicle and AngII-treated mice (Figure 3B), corroborating the higher BP. However, acetylcholine-mediated vasodilation in absence and in presence of L-NIO was reduced by hypertension to the same extent in both groups treated with AngII (Figure 3C and 3D). Western blot analysis of thoracic aortas showed a significant reduction in phosphorylated-vasodilator-stimulated phosphoprotein in basal and AngII-treated ECKO-Spns2 mice versus Spns2$^{f/f}$ (Figure 3E and 3F). Of note, the loss of endothelial Spns2 did not affect S1P-mediated vasodilation of MA (Figure 3G), the S1P receptor expression on the vasculature (Figure 3H and 3I), as well as the response to phenylephrine (Figure S3A), suggesting that the altered vascular functions were because of the loss of endothelial-derived S1P rather than downstream S1P signaling machinery. These data indicate that endothelial S1P sustains local vascular homeostasis by regulating vasodilation in response to shear stress via autocrine S1P-S1P1-NO signaling, with impact on systemic BP.

ApoM Deficiency Exacerbates Hypertension and Cardiac Hypertrophy

We have previously reported that mice lacking ApoM, with half of S1P plasma levels,[26] showed a significant

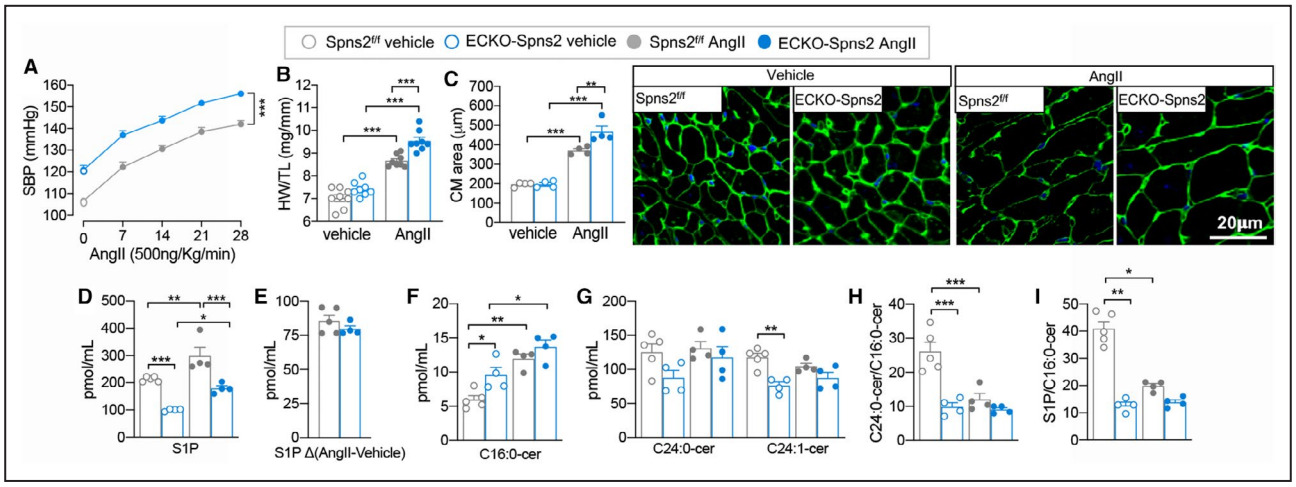

Figure 2. The loss of *Spns2* exacerbates hypertension and cardiac hypertrophy, and correlates with a pathological ceramide and S1P plasma profile.

A, ECKO-Spns2 and Spns2$^{f/f}$ SBP measured with tail-cuff system (n≥8 mice/group) before and 4 weeks after angiotensin II (AngII) osmotic pump implantation. **B**, Heart weight/tibia length (HW/TL) ratios of AngII and vehicle-treated Spns2$^{f/f}$ and ECKO-Spns2 mice (n≥8 mice/group). **C**, Quantification of cardiomyocyte (CM) cross-sectional area and representative immunofluorescent FITC-labeled wheat germ agglutinin and 4'6-diamidino-2-phenylindole–stained heart sections from AngII- and vehicle-treated Spns2$^{f/f}$ and ECKO-Spns2 mice (n=4 mice/group). S1P and ceramides measurements of plasma from AngII- and vehicle-treated Spns2$^{f/f}$ and ECKO-Spns2 mice. **D**, S1P; (**E**) Delta of S1P increase in the plasma of AngII- vs vehicle-treated mice; (**F**) C16:0-ceramide; (**G**) C24:0- and C24:1-ceramide; (**H**) C24:0-/C16:0-ceramide ratio; and (**I**) S1P/C16:0-ceramide ratio. n=5 mice per group. Data are expressed as mean±SEM. *P≤0.05; **P≤0.01; ***P≤0.001. Statistical significance was determined by 2-way ANOVA with Sidak's post hoc test (**A**) and 2-way ANOVA with Tukey's test (**B** through **D** and **F** through **I**). FITC indicates fluorescein isothiocyanate; S1P, sphingosine-1-phosphate; and SBP, systolic blood pressure.

increase in SBP at baseline versus control littermates,[46] suggesting that HDL-ApoM-bound S1P contributes to maintain normal BP. The goal of this current study was to understand whether HDL-ApoM-bound S1P exerts a protective role in hypertension. Following AngII infusion, ApoM KO mice developed a significantly higher hypertension (Figure 4A) as well as cardiac hypertrophy (Figure 4B) compared with ApoM WT. The latter finding was corroborated by the measure of cardiomyocyte cross-sectional area, which was significantly augmented in hypertensive ApoM KO versus ApoM wild type (WT) mice (Figure 4C). These data suggest that ApoM-bound S1P exerts vasculoprotective functions in a pathological setting, with impact on cardiac remodeling.

Loss of ApoM Exacerbates Vascular Dysfunction During Hypertension

ApoM KO mice present endothelial barrier impairment because of the loss of HDL-bound S1P-mediated endothelial functions.[47] However, the impact of ApoM on endothelial-dependent regulation of vascular tone in pathophysiological conditions has yet to be investigated. Although SBP was higher in ApoM KO versus ApoM WT mice, vascular tone regulation by flow and acetylcholine was preserved (Figure 5A and 5B), suggesting that in physiological conditions the loss of ApoM raises SPB without compromising vessel functions. However, hypertensive ApoM KO MA showed a significantly reduced vasorelaxation in response to flow (Figure 5A) and acetylcholine (Figure 5B), while phenylephrine-induced vasoconstriction was not affected (Figure S3B), suggesting that the absence of circulating ApoM-bound S1P during hypertension accelerates the onset of endothelial dysfunction.

Interestingly, these findings were supported by diminished basal but not acetylcholine-stimulated NO production in normotensive and hypertensive ApoM KO MA compared with ApoM WT (Figure 5C and 5D). Lastly, the loss of ApoM did not alter the vasodilation in response to exogenous S1P (Figure 5E). Altogether, these findings corroborate an important role of ApoM-bound S1P in preserving vascular and BP homeostasis.

DISCUSSION

This study investigated the relevance of S1P bound to ApoM of HDL and S1P originated by the endothelium to vascular functions and hypertension. Multiple clinical studies have reported a negative correlation between circulating levels of HDL-S1P and the risk of coronary artery disease and diabetes mellitus.[31,32,48,49] However, the role of HDL-bound S1P in hypertension remains an open question.

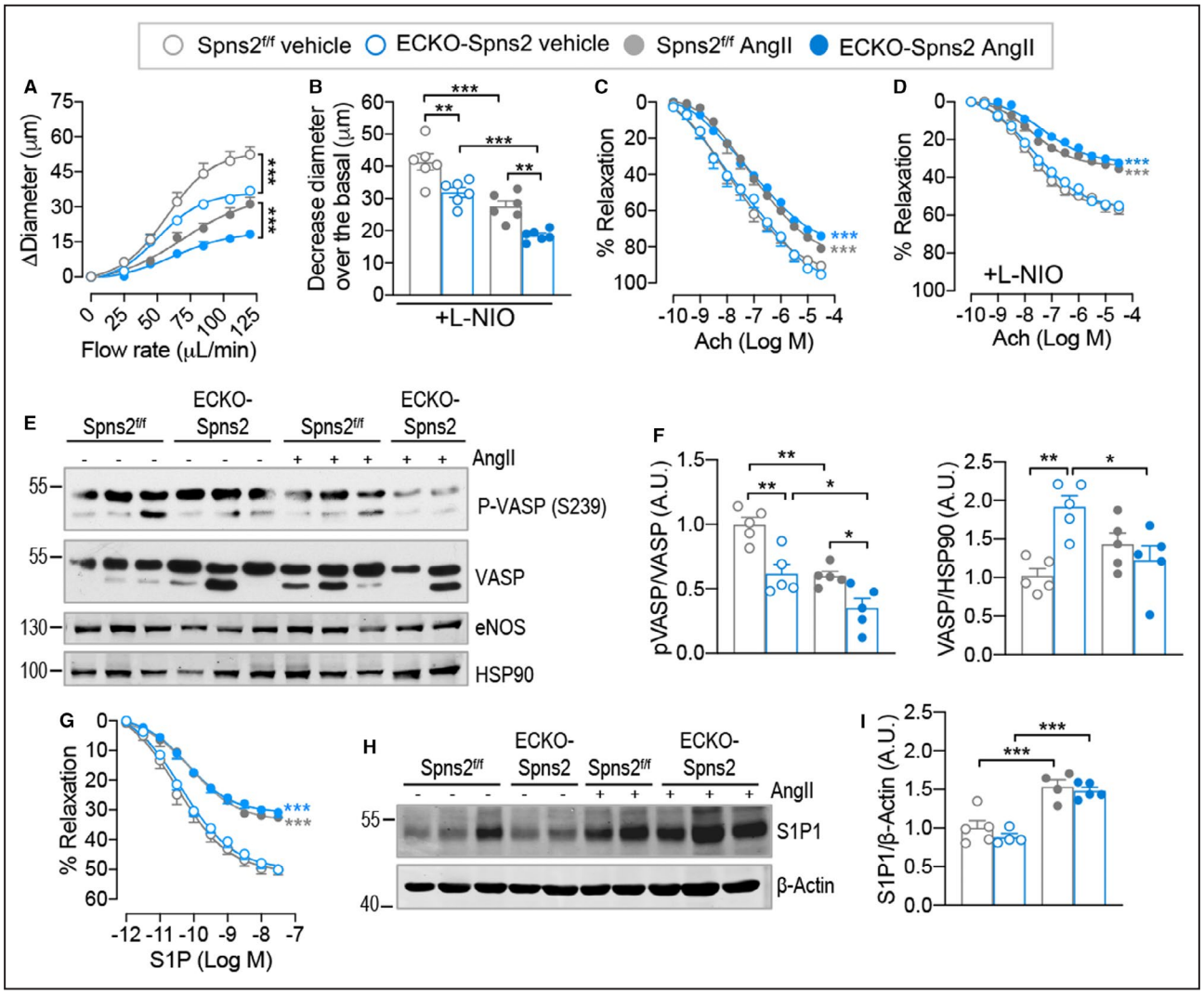

Figure 3. Endothelial S1P-NO signaling mediates flow-induced vasodilation in healthy and hypertensive resistance arteries. Mesenteric arteries (MA) from normotensive and hypertensive Spns2$^{f/f}$ and ECKO-Spns2 mice (n=5/group, n≥8 MA/group) were assessed for vascular reactivity in a pressure myograph system. **A**, Flow-mediated vasodilation. **B**, MA were incubated with L-NIO (100 μmol/L, 15 minutes) and the decrease in luminal diameter compared with baseline was assessed; (**C**) acetylcholine (Ach)-mediated vasodilation and (**D**) Ach-induced vasorelaxation in presence of L-NIO (100 μmol/L). **E**, Western blot (WB) analysis for phosphorylated vasodilator-stimulated phosphoprotein (P-VASP), VASP, and eNOS on ECKO-Spns2 and Spns2$^{f/f}$ thoracic aortas, with or without angiotensin II (AngII) (n=5 mice/group) and (**F**) relative quantification. **G**, Sphingosine-1-phosphate (S1P)-mediated vasodilation. **H**, WB analysis for S1PR1 on ECKO-Spns2 and Spns2$^{f/f}$ thoracic aortas, with or without angiotensin II (n≥4 mice/group) and (**I**) relative quantification. Data are expressed as mean±SEM. *P≤0.05; **P≤0.01; ***P≤0.001; gray asterisks refer to hypertensive vs normotensive Spns2$^{f/f}$; blue asterisks refer to hypertensive vs normotensive ECKO-Spns2. Statistical significance was determined by 2-way ANOVA with Sidak post-test (**A**, **C**, **D**, and **G**) and 1-way ANOVA with Tukey's post-test (**B**, **E**, and **H**). eNOS indicates endothelial nitric oxide synthase; and L-NIO, N5-(1-iminoethyl)-L-ornithine dihydrochloride. heat shock protein 90 (HSP90).

S1P1 is highly expressed in the endothelium and controls blood flow and pressure.[13,50] As previously reported by us and others[13,51] S1P1 is not decreased in hypertension (Figure 3H), and can be activated by agonists, resulting in BP-lowering effects.[22,46] These data suggest that an impairment in ligand bioavailability rather than receptor expression may disrupt S1P signaling in hypertension.

The endothelium is an important source of plasma S1P[18,19] and sustains local autocrine S1P-S1P1-NO signaling.[13,19,22] Increased endothelial S1P production by Nogo-B deletion, a downregulator of sphingolipid de novo biosynthesis, enhances flow-mediated vasodilation,[22] an effect that was eliminated by myriocin, a pharmacological inhibitor of the pathway, pointing to endothelial-derived S1P as an active player in flow-mediated vasodilation. Thus, to understand the direct pathophysiological role of endothelial S1P in hypertension, we used mice lacking Spns2.

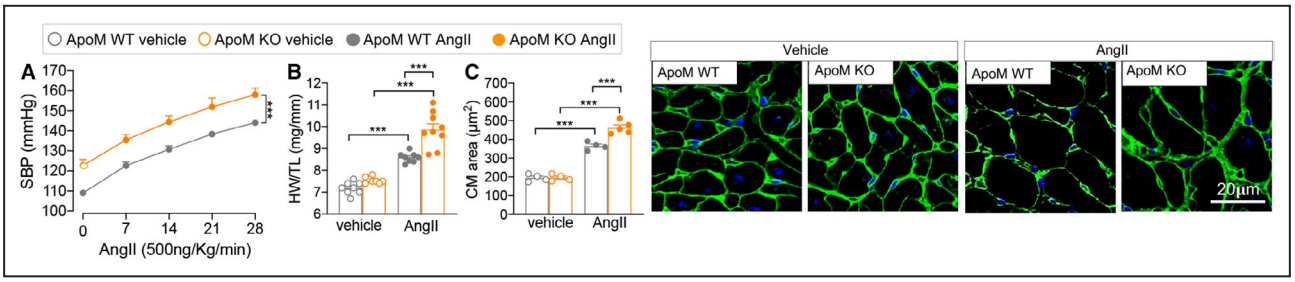

Figure 4. ApoM deficiency exacerbates hypertension and cardiac hypertrophy.
A, SBP was measured with tail-cuff system in ApoM WT and ApoM knock out (KO) mice before and after AngII-osmotic pump implantation once/wk for 28 days (n≥8 mice/group). **B**, Heart weight (mg)/tibia length (mm) (HW/TL) ratios in normotensive and hypertensive ApoM WT and ApoM KO mice (n≥8 mice/group). **C**, Quantification and representative immunofluorescence images of cardiomyocyte (CM) cross-sectional area in vehicle (n=4/group) and Ang-II infused ApoM WT (n=4 mice) and ApoM KO (n=5 mice). Data are expressed as mean±SEM. ***P≤0.001. Statistical significance was determined by 2-way ANOVA with Sidak's post hoc test (**A**) or 2-way ANOVA with Tukey's post-test (**B** and **C**). AngII indicates angiotensin II; ApoM, apolipoprotein M; and SBP, systolic blood pressure.

Our study demonstrates that (1) the loss of both HDL-bound S1P and endothelial-derived S1P heightens BP in normal and hypertensive conditions; (2) the lack of Spns2 is associated with impaired BP dipping; (3) the loss of ApoM and Spns2 exaggerates cardiac hypertrophy in hypertension; (4) Spns2 is necessary to preserve S1P-mediated vasodilation in response to flow; (5) plasma S1P increase is not causal of hypertension; (6) sources other than the endothelium contribute to the increase of plasma S1P in hypertension; and (7) plasma S1P/C16:0-cer rather than S1P levels correlates better with high BP (Figure 6).

In agreement with previous studies,[51,52] plasma S1P is increased in hypertension (Figure 2D). However, despite lowering plasma S1P, the loss of endothelial Spns-2 significantly increased BP at baseline and following AngII infusion, arguing against a pathological role of plasma S1P increase in hypertension, and supporting vasculoprotective functions of endothelial-derived S1P signaling.

S1P gradient orchestrates lymphocytes trafficking.[1] Fukuhara et al reported that in mice lacking Spns-2, plasma S1P and circulating lymphocytes are markedly reduced.[20] Lymphocytes play an important role in the pathogenesis of hypertension.[53-55] Nonetheless, BP was significantly upregulated in both physiological and pathological states, suggesting that the loss of endothelial-derived S1P overcomes the reduction in circulating lymphocytes. Similar conclusions were reached by chronic administration of fingolimod, a functional antagonist of S1P1, approved for the treatment of relapsing multiple sclerosis.[56] Although reducing circulating lymphocytes, fingolimod significantly increased BP in healthy and hypertensive mice, mainly by suppressing S1P1 signaling in the vasculature.[13] Indeed, fingolimod recapitulated the hypertensive phenotype reported in mice lacking endothelial S1P1,[13] underlying a critical and necessary role for S1P-S1P1 signaling in preserving vascular health.

Our study also uncovers a novel role of endothelial-derived S1P signaling, which is preserving the circadian pattern of BP. In addition to elevated BP, ECKO-Spns2 manifested a reduced decrease in BP during the day, uncovering an impaired dipping phenotype. Nondipping pattern is associated with high risk for cardiovascular events, end organ damage, and poor prognosis.[57,58] Hence, our results reinforce the concept that endothelial-derived S1P signaling is necessary to preserve BP and its circadian pattern.

As a potent activator of eNOS,[11] S1P contributes to lower vascular tone and BP mainly via S1P1-NO signaling.[13] As is the case in the absence of S1P1,[13] flow-mediated vasodilation was blunted in ECKO-Spns2, underscoring the importance of endothelial-derived S1P in activating S1P1 to induce vasorelaxation in response to flow. Furthermore, NO production and the phosphorylation of its downstream target vasodilator-stimulated phosphoprotein were significantly reduced in normotensive and hypertensive ECKO-Spns2 mice, corroborating the concept that the disruption of the endothelial S1P-S1P1-eNOS autocrine signaling contributes to vascular dysfunction by impairing tonic and flow-mediated eNOS signaling.

Plasma ceramide ratios, particularly C24:0-cer/C16:0-cer, negatively correlated with mortality in patients affected by coronary artery disease.[38,44,45] Recently, we demonstrated that the endothelium is an important source of plasma ceramides and can be considered as a "recounter" of the vascular disease state.[19] Interestingly, similarly to patients with coronary artery disease, C24:0-cer/C16:0-cer ratio is significantly lower in AngII-infused mice, as well as in ECKO-Spns2 mice, in line with the high BP. Our data also showed that S1P/C16:0-cer ratio is markedly decreased in hypertension as well as in ECKO-Spns2, presenting vascular dysfunction and elevated BP already at baseline.

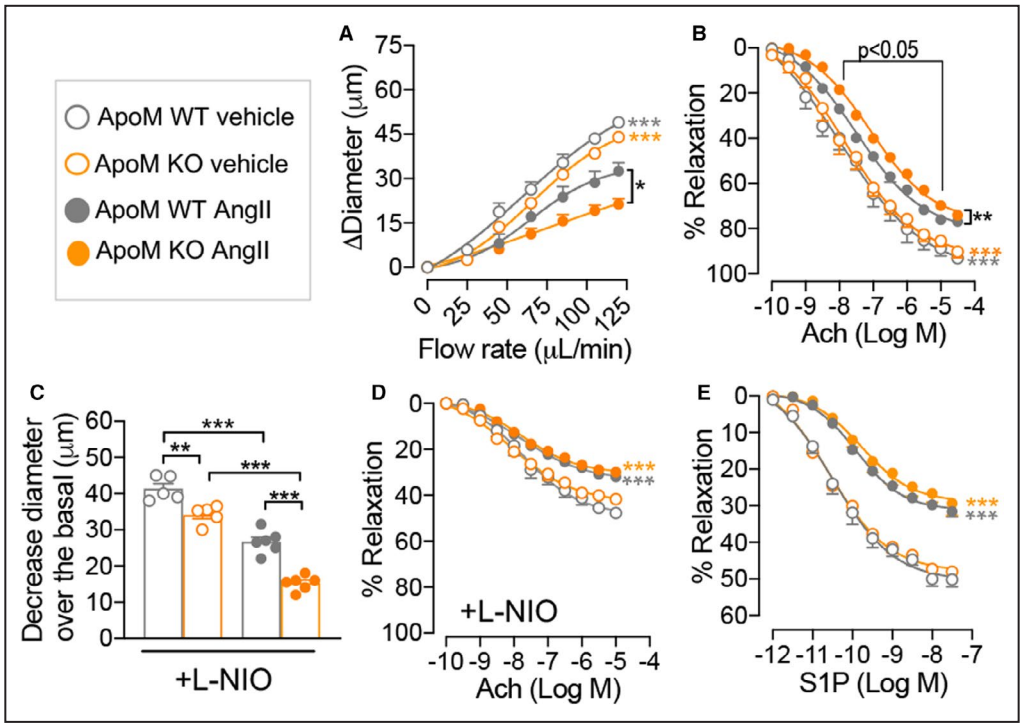

Figure 5. ApoM-bound S1P preserves vascular functions in hypertension.
Mesenteric arteries (MA) from ApoM WT and ApoM KO mice (n=5/group, n≥8 MA/group) were assessed for vascular reactivity in a pressure myograph system. **A**, Flow- and (**B**) acetylcholine (Ach)-induced vasorelaxation. *P*<0.05 indicates the significance determined by 2-way ANOVA with Sidak post-test. **C**, MA were incubated with L-NIO (100 μmol/L, 15 minutes) and the decrease in luminal diameter compared with baseline was measured. **D**, Ach-induced vasorelaxation in presence of L-NIO. **E**, S1P-mediated vasodilation. Data are expressed as mean±SEM. **P*≤0.05; ***P*≤0.01; ****P*≤0.001. Gray asterisks refer to hypertensive vs normotensive ApoM WT; orange asterisks refer to hypertensive vs normotensive ApoM KO. Statistical significance was determined by 2-way ANOVA with Sidak's post-test (**A**, **B**, **D**, and **E**) and 2-way ANOVA with Tukey's post-test (**C**). AngII indicates angiotensin II; ApoM, apolipoprotein M; KO, knock out; L-NIO, N5-(1-iminoethyl)-L-ornithine dihydrochloride; S1P, sphingosine-1-phosphate; and WT, wild type.

Thus, the circulating S1P/C16:0-cer ratio might better correlate with the pathological state of the vasculature.

Most of the S1P in human plasma is associated with ApoM of HDL.[26] S1P bound to HDL decreases in different cardiovascular diseases, including myocardial infarction and coronary artery diseases.[31,32,59] In hypertension, plasma S1P is increased in humans and mice[51,60] (Figure 2), although the relative abundance of different circulating S1P pools is yet to be defined. Furthermore, S1P bound to ApoM-HDL exerts prevailing anti-inflammatory and anti-atherosclerotic functions on the endothelium compared with albumin-bound S1P, by acting as biased agonist.[61]

The loss of ApoM exacerbates vascular permeability,[26,36] inflammation, and atherosclerosis,[61] whereas elevated levels of ApoM could slow the progression of the disease.[62] While the role of ApoM has been studied in atherosclerosis, its function in hypertension remains unknown. Our study demonstrates, for the first time, that ApoM of HDL is a key player in BP regulation, most likely via S1P. Mice lacking ApoM present high BP at baseline as well as following AngII infusion, most likely because of the loss of ApoM-bound S1P vasorelaxation and BP-lowering effects. In physiological conditions, the overall vascular reactivity was preserved, except for basal NO production, which was impaired. Interestingly, in AngII-induced hypertension, flow- and acetylcholine-mediated vasodilation was significantly reduced in ApoM KO mice, underscoring the protective functions of ApoM-bound S1P on the endothelium in pathological conditions.

Our study demonstrates that both endothelial-derived and ApoM-bound S1P protect the heart from pathological cardiac hypertrophy in hypertension. Hemodynamic stress from higher BP can promote cardiac hypertrophy and dysfunction, leading to heart failure.[63] However, it is conceivable that additional molecular mechanisms, other than lowering BP, contribute to the S1P cardioprotective functions, especially considering that endothelial-derived S1P could target receptors on nearby cells other than the endothelium. Various studies have reported direct beneficial effects

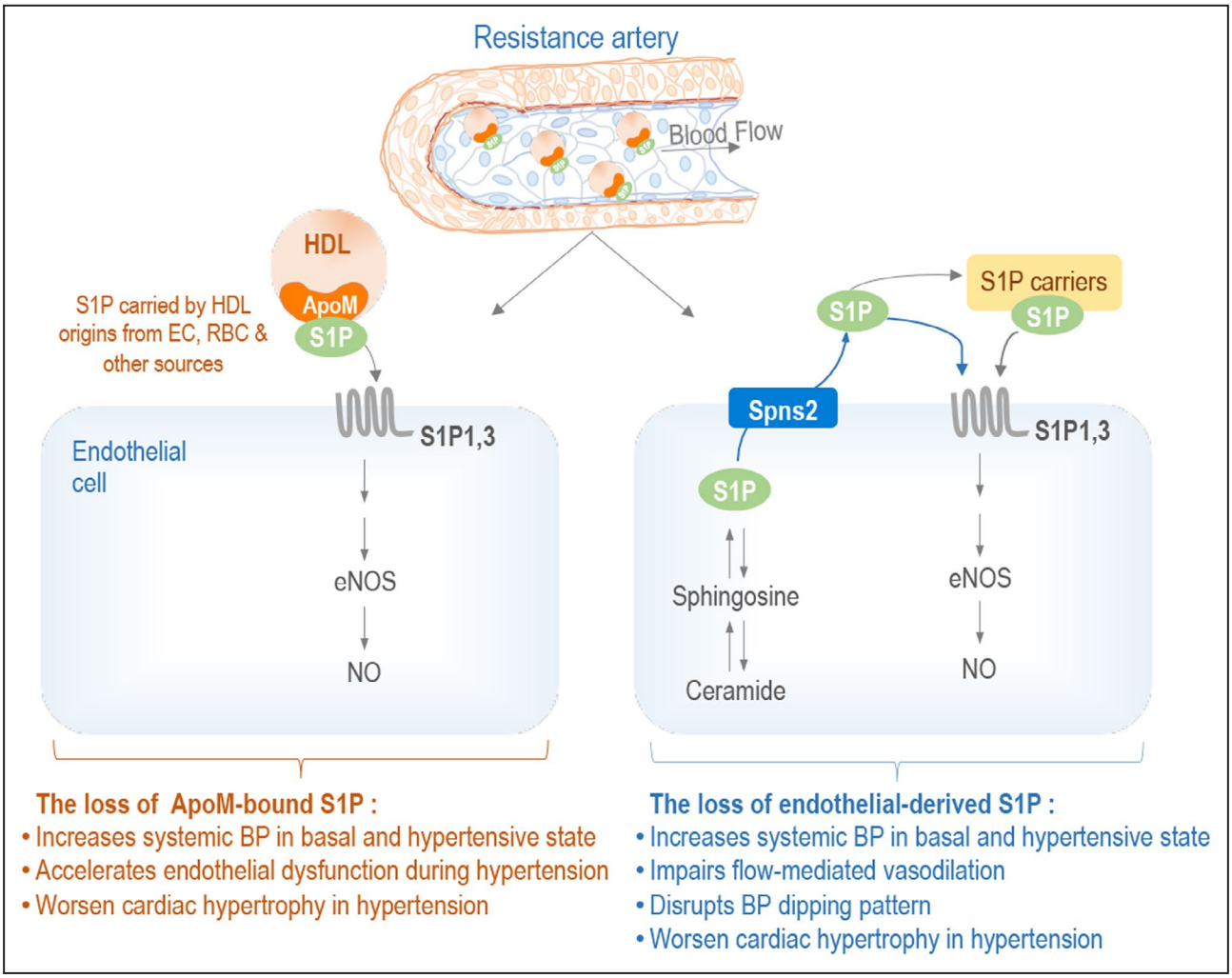

Figure 6. Proposed model.
Both endothelial-derived and ApoM-bound S1P maintain BP homeostasis. The loss of ApoM (left) and Spns2 (right) results in increased BP at baseline and following chronic infusion of AngII because of the disruption of S1PR1,3-NO signaling lowering vascular tone. Endothelial Spns2 is necessary to mediate the vasodilation in response to flow via S1P-NO signaling and preserve BP dipping phenotype. Derangement of both S1P pools impair systemic BP regulation and cardiac remodeling in hypertension. ApoM indicates apolipoprotein M; BP, blood pressure; EC, endothelial cells; eNOS, endothelial nitric oxide synthase; HDL, high-density lipoprotein; RBC, red blood cells; S1P, sphingosine-1-phosphate; and Spns2, spinster 2 transporter.

of S1P on the heart.[46,64-66] Recently, Keul and coworkers demonstrated that the lack of S1P1 in cardiomyocyte leads to cardiomyopathy, heart fibrosis, and premature death.[67] Mice lacking endothelial Nogo-B (which downregulates S1P synthesis) were protected from myocardial permeability, inflammation, and dysfunction induced by pressure overload.[23] A recent study revealed a cardioprotective role of ApoM-bound S1P via myocardial autophagy.[68] Altogether, published and current findings of this study suggest that S1P signaling can protect the heart from pathological hypertrophy by different means.

Our findings have great potential for translation. HDL-bound S1P are reduced in atherosclerosis, coronary artery diseases, myocardial infarction,[31-33] and heart failure.[69] The correlation between *APOM* single nucleotide polymorphism and atherosclerosis[34,35] underscores its clinical relevance. Recently, we reported that engineered ApoM-bound S1P can lower BP and protect from myocardial infarction,[46] demonstrating its therapeutic potentials.

In conclusion, our study reveals the importance of S1P signaling in hypertension and cardiac hypertrophy. Specifically, both HDL-bound and endothelial-derived S1P preserve vascular functions and BP via NO signaling. Thus, derangements of both S1P pools can undermine the health state of the vasculature with pathological implications for the heart. In addition, in regard to regulating blood flow, we have identified a novel role for endothelial-derived S1P in preserving BP dipping. This finding is clinically relevant because a

nondipper BP pattern correlates with increased risk for a cardiovascular event. Finally, our study also shows that the dysregulation of endothelial sphingolipid metabolism is mirrored by plasma S1P and ceramide profile, which might be indicative of the health/disease state of the vasculature. By providing a therapeutic framework, our findings have clinical implications not only in hypertension but also in cardiovascular and metabolic disorders, characterized by deranged sphingolipid homeostasis and signaling.

ARTICLE INFORMATION

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Disclosures

Drs Hla and Di Lorenzo report to have a patent for Apom-fc fusion proteins, complexes thereof with S1P and methods for treating vascular and nonvascular diseases. The remaining authors have no disclosures to report.

Supplementary Material

Figures S1–S3

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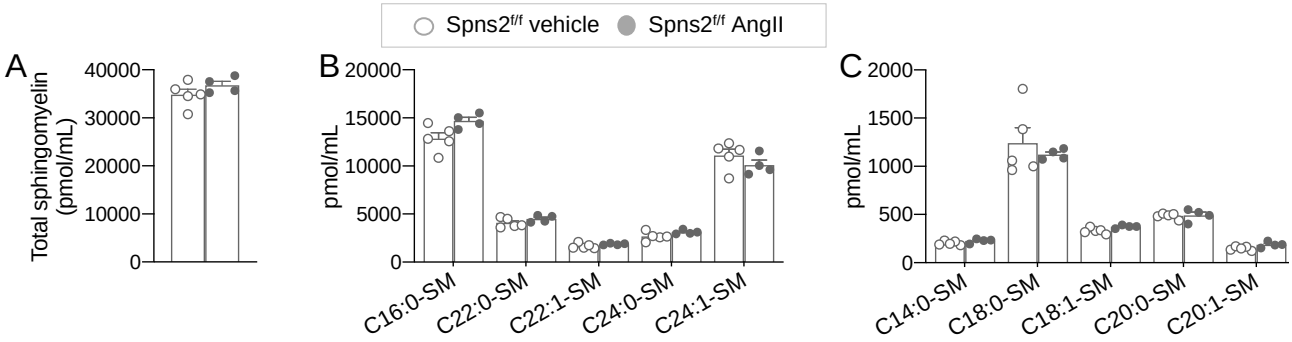
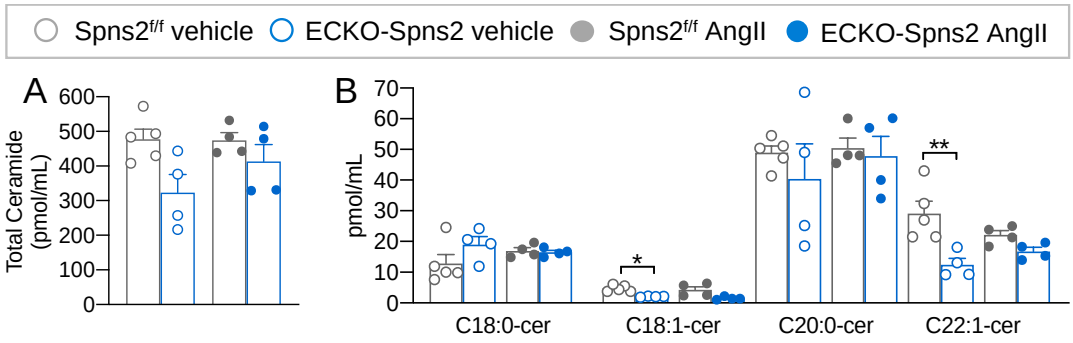
SUPPLEMENTAL MATERIAL

Figure S1. Plasma levels of sphingomyelin (SM) do not change in hypertension.



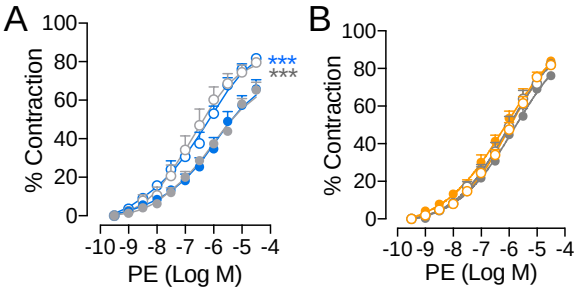
Plasma measurements of SM from normotensive and hypertensive Spns2$^{f/f}$ mice. n ≥ 4 mice per group. **(A)** Total SM; **(B)** C16:0-, C22:0-, C22:1-, C24:0-, C24:1-SM; **(C)** C14:0-, C18:0-, C18:1-, C20:0-, C20:1-SM. Data are expressed as mean ± SEM. Statistical significance was determined by Unpaired t-test.

Figure S2. Ceramide measurements in plasma from normotensive and hypertensive Spns2f/f and ECKO-Spns2 mice.



(A) Total ceramide; **(B)** C18:0-, C18:1-, C20:0-, C22:1-ceramide; n ≥ 4 mice per group. Data are expressed as mean ± SEM. **P < 0.01. Statistical significance was determined by Two-way ANOVA with Tukey post-test.

Figure S3. Concentration-response to PE.



MA from normotensive and hypertensive mice were assessed for PE-mediated contraction in a pressure myograph system. (**A**) Spns2$^{f/f}$ and ECKO-Spns2 mice (n=5/group, n≥8 MA/group), and (**B**) ApoM WT and ApoM KO mice (n=5/group, n≥8 MA/group). Data are expressed as mean ± SEM; ***P ≤ 0.001. Grey asterisks refer to hypertensive vs normotensive Spns2$^{f/f}$ and ApoM WT; blue asterisks refer to hypertensive vs. normotensive ECKO-Spns2; orange asterisks refer to hypertensive vs. normotensive ApoM KO. Statistical significance was determined by Two-way ANOVA with Sidak's post-test.