

Heart

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Cardiovascular Effects of Pharmacological Targeting of Sphingosine Kinase 1

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Abstract—High blood pressure is a risk factor for cardiovascular diseases. Ang II (angiotensin II), a key pro-hypertensive hormone, mediates target organ consequences such as endothelial dysfunction and cardiac hypertrophy. S1P (sphingosine-1-phosphate), produced by Sphk1 (sphingosine kinase 1), plays a pivotal role in the pathogenesis of hypertension and downstream organ damage, as it controls vascular tone and regulates cardiac remodeling. Accordingly, we aimed to examine if pharmacological inhibition of Sphk1 using selective inhibitor PF543 can represent a useful vasoprotective and cardioprotective anti-hypertensive strategy in vivo. PF543 was administered intraperitoneally throughout a 14-day Ang II-infusion in C57BL6/J male mice. Pharmacological inhibition of Sphk1 improved endothelial function of arteries of hypertensive mice that could be mediated via decrease in eNOS (endothelial nitric oxide synthase) phosphorylation at T495. This effect was independent of blood pressure. Importantly, PF543 also reduced cardiac hypertrophy (heart to body weight ratio, 5.6 ± 0.2 versus 6.4 ± 0.1 versus 5.9 ± 0.2 mg/g; $P < 0.05$ for Sham, Ang II+placebo, and Ang II+PF543-treated mice, respectively). Mass spectrometry revealed that PF543 elevated cardiac sphingosine, that is, Sphk1 substrate, content in vivo. Mechanistically, RNA-Seq indicated a decreased expression of cardiac genes involved in actin/integrin organization, S1pr1 signaling, and tissue remodeling. Indeed, downregulation of Rock1 (Rho-associated coiled-coil containing protein kinase 1), Stat3 (signal transducer and activator of transcription 3), PKC (protein kinase C), and ERK1/2 (extracellular signal-regulated kinases 1/2) level/phosphorylation by PF543 was observed. In summary, pharmacological inhibition of Sphk1 partially protects against Ang II-induced cardiac hypertrophy and endothelial dysfunction. Therefore, it may represent a promising target for harnessing residual cardiovascular risk in hypertension. (*Hypertension*. 2020;75:383-392. DOI: 10.1161/HYPERTENSIONAHA.119.13450.) • [Online Data Supplement](#)

Key Words: angiotensin II ■ blood pressure ■ cardiac hypertrophy ■ hypertension ■ sphingosine 1-phosphate ■ sphingolipids

High blood pressure (BP) remains an important clinical problem, as it is a major risk factor for a variety of cardiovascular diseases.¹ However, while for years we have been focusing on BP as a main cardiovascular disease symptom, it has become apparent that high BP carries a particularly high residual risk linked to both vascular and cardiac dysfunction and is linked to multi-organ hypertensive pathology.

Ang II (angiotensin II), affects a broad range of processes, such as salt and water retention or elevation of vascular contractility and resulting peripheral arterial resistance,^{2,3} all contributing to BP increase. Chronically elevated BP leads, in time, to progressive target-organ damage, for example, in the heart manifesting left ventricular (LV) hypertrophy, myocardial ischemia, and heart failure.⁴

S1P (sphingosine-1-phosphate), a bioactive lysophospholipid produced by Sphks (sphingosine kinases), is a regulator of cellular processes such as proliferation,⁵ migration,⁶ and

apoptosis.⁷ Experimental hypertension results in elevated plasma S1P level,^{8,9} and our, as well as other, studies demonstrated protection of *Sphk1* knockout mice against development of Ang II-induced hypertension.⁸⁻¹⁰ Furthermore, it has been shown that mice lacking *S1pr2* (*sphingosine-1-phosphate receptor 2*) exhibit reduced peripheral vascular resistance as a result of impaired vasoconstrictor responses.¹¹ However, mice lacking Nogo-B, a transcription factor which inhibits the rate-limiting step of de novo sphingolipid synthesis, are hypotensive and resistant to hypertension in response to Ang II due to autocrine S1P signaling in endothelium preserving endothelial function.¹² In addition, S1pr1 was identified as a key positive regulator of flow-mediated endothelial vasodilation, and EC-specific *S1pr1* downregulation was correlated with increased BP.¹³

Studies on the cardiac role of S1P/Sphk1 revealed that S1P affects cardiac contractility and heart rate, plays an important role in cardioprotection in response to ischemic injury,

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and regulates cardiac hypertrophy and fibrosis.¹⁴ In vitro studies have demonstrated that downregulation of Sphk1 signaling inhibits TGF- β (transforming growth factor- β)-stimulated collagen production in mouse cardiac fibroblasts.¹⁵ Furthermore, stimulation of rat neonatal cardiomyocytes with S1P led to cell growth in size, and this effect was abolished by S1pr1 antibody treatment,¹⁶ while mice overexpressing *Sphk1* developed spontaneous myocardial degeneration and cardiac fibrosis.¹⁷ Moreover, it was shown that cardiac fibroblast-specific overexpression of *S1pr1* in mice increases hypertrophy and fibrosis of heart tissue which is accompanied by upregulation in Stat3 (signal transducer and activator of transcription 3) signaling and IL-6 (interleukin-6) production.¹⁸ Interestingly, our previous study showed that deletion of *Sphk1* protects against Ang II–induced cardiac hypertrophy.⁸

The above studies suggest that pharmacological modulation of S1P/Sphk1 signaling may be of interest in the context of cardiovascular research. Therefore, the goal of this study was to define the effect of pharmacological modulation of Sphk1 activity on the development of Ang II–dependent systemic arterial hypertension and associated vascular dysfunction as well as cardiac hypertrophy by using selective Sphk1 inhibitor—PF543 in vivo.

Methods

An extended description of the methods is available in the [online-only Data Supplement](#).

Data Availability Statement

Raw gene counts and final results of the RNA-Seq analysis are available as Table in the [online-only Data Supplement](#). Other data that support the findings of this study are available from the corresponding author on reasonable request.

Induction of Hypertension and PF543 Treatment In Vivo

Male C57BL6/J mice at the age of 12 to 14 weeks bred in specific pathogen-free facility, fed with standard chow, and randomly assigned to the control and treatment groups were investigated. Hypertension was induced by 14-day infusion of Ang II (490 ng/kg per minute, Sigma-Aldrich) using subcutaneously implanted osmotic minipump (Alzet) following intraperitoneal anesthesia with Ketamine (100 mg/kg)/xylazine (10 mg/kg) solution (both Biowet, Poland). Two models of PF543 treatment were tested: (1) a rescue model—a single intraperitoneal injection with PF543 (Cayman Chemical) at a dose of 10 mg/kg (dissolved in 20% β -hydroxypropyl-cyclodextrin in PBS) of normotensive mice or hypertensive mice (on the 13th day of continuous Ang II infusion) and (2) a chronic model—administered PF543 intraperitoneally every 2 days (at a dose of 1 or 10 mg/kg) commencing the day before implantation of the Ang II–dosed pump. Importantly, MacRitchie et al¹⁹ demonstrated that application of the higher PF543 dose (ie, 10 mg/kg) degrades Sphk1 in pulmonary vessels in mice,¹⁹ while Zhang et al²⁰ found that lower, 1 mg/kg, dose of PF543 inhibits murine cardiac sphingosine kinase activity and lowers serum S1P content. Mice underwent noninvasive systolic BP measurement by tail-cuff plethysmography (Visitech BP 2000 BP Analysis System) before commencement of the treatment and during hypertension development. After 2 weeks of Ang II infusion, mice were euthanized, tissues were collected and subjected to subsequent experiments. For RNA and protein isolation, tissues were lysed in dedicated buffers²¹ (see [online-only Data Supplement](#) for details). If possible, experiments were performed on blinded samples. All experiments were approved by the II Local Ethics Committee in Cracow (approval number 157/2016).

RNA-Seq Analysis

To recapitulate possible changes in transcriptome profile caused by downregulation of S1pr1, we studied LV samples obtained from hypertensive mice treated either with 10 mg/kg per 2 days PF543 dose or placebo. Total RNA from the LV of hypertensive mice, chronically treated with either PF543 (intraperitoneal 10 mg/kg every 2 days; n=4) or solvent control (n=4), was isolated using Direct-zol RNA Miniprep kit and treated with DNase I (Zymo Research). Briefly, mRNA was isolated using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). The mRNA library was prepared using NEBNextUltra II Directional RNA Library Prep Kit for Illumina. Sequencing was performed with HiSeq4000 (Illumina) with 150 bp paired-end reads (PE150). Bioinformatic analysis included trimming of adapters, polyA, and polyT tails using Cutadapt.²² RNA-seq reads were then mapped to genes according to the reference genome (*Mus musculus* M20, GRCm38.p6 downloaded from GENCODE) using TopHat software.²³ Reads were counted with HTseq.²⁴

Histological Analysis of Cardiac Tissues

Formalin-fixed and paraffin embedded 7 μ m sections were deparaffinized and rehydrated. Sections were stained with Weigert's iron hematoxylin solution (Sigma-Aldrich) for 10 minutes and then washed and incubated in the dark with 0.1% Sirius red F3B (Sigma-Aldrich) for 1 hour, washed in acidified water, dehydrated, and mounted. Additionally, fixed tissue was also stained with Masson's trichrome (Sigma-Aldrich, Germany) according to the manufacturer protocol. The cross-sectional cardiomyocyte size was calculated using cells with integral membrane and visible nucleus (\approx 60 cells per section). Relative fibrotic area and cardiomyocyte size quantification was performed using ImageJ (version 1.49) software.²⁵

Measurement of Cardiac Sphingosine Content

Heart tissues were homogenized and subjected to lipid extraction by a modified 1-step Bligh and Dyer protocol.^{26,27} Then, samples underwent chemical methylation.^{28,29} Samples were analyzed in positive ion mode using a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a TriVersaNanoMate (Advion Biosciences, Ithaca, NY). The acquisition cycle consisted of Fourier-transform mass spectrometry (FT MS) and targeted Fourier-transform tandem mass spectrometry (FT MS/MS) scans in the positive mode. Acquired data were processed by using LipidXplore software.³⁰ More details are provided in the [online-only Data Supplement](#).

Statistical Analysis

Differential gene expression analysis from the RNA-Seq experiment was performed using DESeq2 package in R (version 3.5.1),³¹ while Overrepresentation Enrichment Analysis was performed using WebGestalt³² and all 599 genes with false discovery rate (FDR) corrected *P* value <0.05 (Table in the [online-only Data Supplement](#) for a summary statistics of all genes detected in the RNA-Seq analysis). To check whether and how chronic PF543 treatment affected cardiac processes related to cell death, we performed overrepresentation analysis separately for genes significantly (FDR, *P*<0.05) up- or downregulated. Repeated measures ANOVA was used for analysis of vascular function studies and BP measurements, and *t* tests were used for analysis of mRNA/protein expression data. All these statistical tests were performed using IBM SPSS Statistics (version 25) or GraphPad Prism (version 7). Data are presented as mean \pm SEM. *P* values <0.05 were considered statistically significant.

Results

Effects of PF543 Treatment on the Development of Ang II–Dependent Hypertension and Associated Cardiac Remodeling

The results revealed that PF543 did not affect systolic BP level during chronic administration in mice developing hypertension using either 1 or 10 mg/kg per 2 days doses (Figure 1A) or 24 hours after drug injection when hypertension was already established (Figure S1A). Similarly, single PF543 injection did

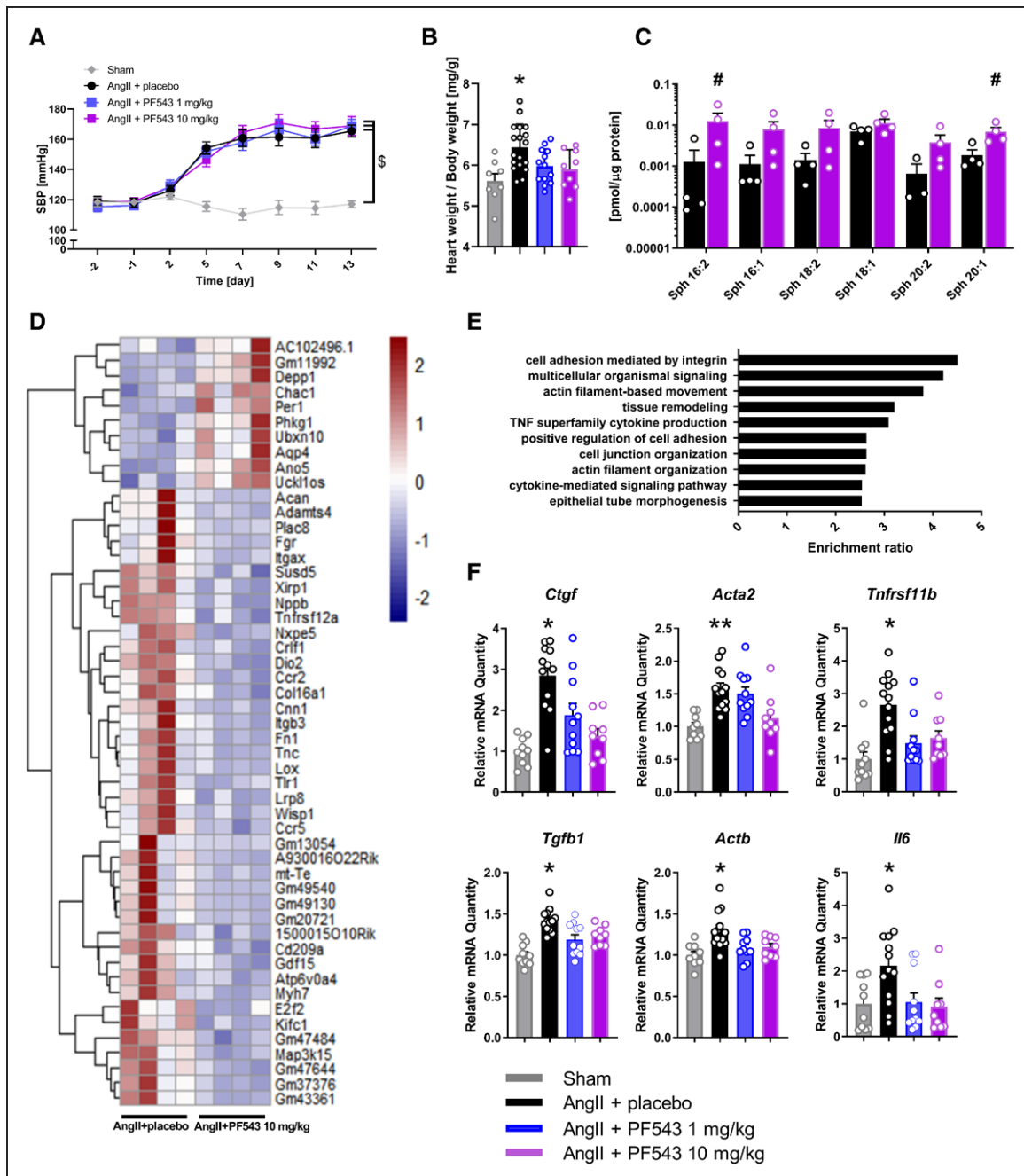


Figure 1. Effects of chronic PF543 treatment on the development of Ang II (angiotensin II)-induced hypertension and cardiac hypertrophy. **A**, Systolic blood pressure (SBP) of Sham or Ang II-infused (490 ng/kg per min) mice treated either with PF543 (1 mg/kg or 10 mg/kg every 2 days) or placebo (solvent). n=8, 17, 13, 11 for Sham, Ang II+placebo, Ang II+PF543 1 mg/kg, Ang II+PF543 10 mg/kg group, respectively. **B**, Heart to body weight ratio (mg/g) of Sham or Ang II-infused (490 ng/kg per min) mice treated either with PF543 (1 or 10 mg/kg every 2 days) or placebo. n=8–18/group. **C**, Measurement of different sphingoid bases content, normalized to total protein concentration, in the left ventricular heart tissue of Ang II-infused mice treated either with PF543 (10 mg/kg every 2 days) or placebo, using mass spectrometry. n=4/group. **D**, Heatmap of 51 genes significantly up- or downregulated (an absolute fold change higher than 1.8) in the left ventricular heart tissue of Ang II-infused mice treated either with PF543 (10 mg/kg every 2 days) or placebo. Transcriptome analysis was performed using RNA-Seq method. n=4/group. **E**, Results of overrepresentation enrichment analysis based on 599 genes with significantly changed expression in the left ventricular heart tissue of Ang II-infused after chronic PF543 (10 mg/kg every 2 days) treatment. **F**, Expression of cardiac hypertrophy-related genes was quantified using real-time polymerase chain reaction in the LV of Sham and Ang II-infused mice treated either with PF543 (1 or 10 mg/kg every 2 days) or placebo. n=9 to 13/group. \$P<0.05 as indicated, *P<0.05 vs all other groups, **P<0.05 vs Sham or Ang II+PF543 10 mg/kg groups, and #P<0.05 vs Ang II+placebo group.

not change blood pressure level of normotensive mice (Figure S1A). Interestingly, chronic PF543 treatment in hypertensive mice significantly reduced cardiac mass, suggesting that Sphk1 may play a role in the regulation of cardiac remodeling in response to Ang II-dependent hypertension (Figure 1B).

Molecular Mechanisms of Cardiac PF543 Action

Using mass spectrometry analysis, we found that the level of possible S1P precursors, that is, sphingoid bases (16:2 and 20:1 species) was significantly elevated in the LV of PF543-treated as compared with placebo-treated hypertensive

animals (Figure 1C), which confirms that PF543 changes sphingolipid balance in the heart. This may have global consequences with respect to the cardiac transcriptome in hypertension. To address this, we performed RNA-Seq analysis, which identified 599 genes significantly up- or downregulated in response to PF543 in LV tissue (Table in the [online-only Data Supplement](#)), including 51 with an absolute fold change higher than 1.8 (Figure 1D). RNA-Seq analysis demonstrated a significant, PF543-mediated downregulation of expression of genes such as *fibronectin 1* (*Fn1*), *integrin subunit α -5* (*Itga5*) and *integrin subunit β -3* (*Itgb3*), *connective tissue growth factor* (*Ctgf*), *actinin α -1* (*Actn1*) or fetal genes *myosin heavy chain 7* (*Myh7*), and *natriuretic peptide B* (*Nppb*; Table in the [online-only Data Supplement](#)).

Subsequent pathway analysis revealed that PF543, among other processes, primarily affected actin filament organization and binding, TNF (tumor necrosis factor) superfamily cytokine production, and tissue remodeling in hypertensive animals (Figure 1E). Real-time polymerase chain reaction experiments confirmed that PF543 treatment reduced mRNA expression of genes from the above pathways accompanying cardiac remodeling such as *Ctgf*, *Tgfb β 1*, α -smooth muscle actin 2 (*Acta2*), β -actin (*Actb*), *interleukin-6* (*Il-6*), and *TNF receptor superfamily member 11b* (*Tnfrsf11b*) in hypertensive animals (Figure 1F).

None of the pathways related to necrosis, apoptosis or autophagy were significantly (FDR, $P < 0.05$) enriched while analyzing genes up- or downregulated by PF543 treatment. We identified 4 pathways related to apoptosis that were enriched among genes significantly downregulated by PF543 treatment at FDR $P < 0.2$ (Figure S2A). Additionally, Western blot analysis showed that chronic PF543 treatment did not change protein level of the apoptotic marker, that is, cleaved form of PARP (poly [ADP-ribose] polymerase; Figure S2A).

Given the fact that RNA-Seq analysis revealed that genes and pathways related to cardiac hypertrophy and fibrosis were altered after PF543 treatment (eg, collagen genes [*Col1a1* and *Col3a1*] were downregulated (unadjusted $P < 0.05$, Table in the [online-only Data Supplement](#)), we performed histological examination of cardiac sections. However, this analysis showed no significant effects of PF543 treatment on cardiac fibrosis in Ang II-infused mice (Figure 2A). On the contrary, PF543 treatment significantly reduced cardiomyocyte size in hypertensive animals (Figure 2B). Additionally, protein quantification by Western blot confirmed a significant upregulation of hypertrophic markers- Acta2 and Fn1 after Ang II infusion, and this effect was attenuated by PF543 treatment (Figure 2C).

PF543 Modulates Key Signaling Pathways Involved in Cardiac Hypertrophy

Ang II treatment significantly induced expression of cardiac *S1pr1* and *S1pr2*, but not *S1pr3*, as compared with Sham animals (Figure S1B). Notably, an initial, rescue experiment testing an acute effect of single PF543 injection demonstrated significantly decreased type 1 S1P-receptor (*S1pr1*), but not *S1pr2* or *S1pr3* mRNA expression in cardiac tissue 24 hours after drug injection (Figure S1B), which was confirmed by

Western blot analysis (Figure S1C). Such decrease in *S1pr1* expression may be regarded as specific for hypertensive conditions as in normotensive mice single injection with PF543 did not affect *S1pr1* protein level (Figure S1D).

Similarly, compared with a single PF543 injection, we found that chronically administered PF543 significantly reduced *S1pr1* protein expression level in the heart (Figure 3A). Interestingly, no significant effects on mRNA expression of *S1pr1* or other S1P receptors in hypertensive mice were observed (Figure 3B). Additionally, although chronic PF543 administration reduced mRNA levels of *Sphk1* in hypertensive animals (Figure S3A), it did not change *Sphk1* protein levels (Figure S3B). We found that *Sphk* activity was significantly increased in cardiac tissue of hypertensive mice as compared with Sham, while chronic PF543 treatment (10 mg/kg) decreased enzyme activity in hypertensive hearts (Figure S3C).

To investigate the mechanism of PF543 action in hypertrophic heart in more detail, we examined expression level of proteins potentially involved in cardiac S1P signaling. Western blot analyses demonstrated that in the hypertrophic heart of Ang II-infused mice there was a significant upregulation of Rock1 (Rho-associated coiled-coil containing protein kinase 1) and phosphorylated forms of Stat3, PKC (protein kinase C) and ERK1/2 (extracellular signal-regulated kinases 1/2) signaling molecules as compared with Sham animals (Figure 4). Importantly, chronic PF543 treatment prevented the above changes in hypertensive animals (Figure 4). In contrast, chronic PF543 treatment in hypertensive mice did not affect the phosphorylation pattern of Akt and GSK3 β (glycogen synthase kinase 3- β) signaling kinases (Figure S2B).

Independent experiments on LV tissue derived from normotensive and Ang II-infused hypertensive or Ang II-infused hypertensive mice treated either with PF543 (10 mg/kg per 2 days) or placebo supported inhibitory effect of PF543 treatment in vivo on the Ang II-induced increment in cardiac Rock1 and *S1pr1* expression, as well as activation status of Stat3 and ERK1/2 (Figure S4; Figure S5 for a wide version of Western blot images of all cardiac proteins analyzed).

Effects of PF543 Treatment on the Ang II-Dependent Vascular Dysfunction In Vivo

Ex vivo tests revealed that adding 10 μ mol/L to the myograph chamber does not constrict mesenteric arteries derived from normotensive mice. Instead, such arteries, precontracted with U-46619, relax in response to increasing doses of PF543 (Figure S6A). Subsequent experiments involving in vivo administration of PF543 demonstrated no changes in contractile responses of mesenteric arteries of hypertensive mice after chronic PF543 treatment (Figure S6B). However, chronic administration of *Sphk1* inhibitor significantly improved acetylcholine-, but not sodium nitroprusside-, mediated vasorelaxation of such arteries (Figure S6C). The above effect was accompanied by a significantly decreased expression of phosphorylated forms of eNOS (T495) and ERK1/2 (T202/Y204; Figure S7A), while no changes in Rock1 protein level or in phosphorylation of PKC at Serine(S) 660 after PF543 treatment in mesenteric arteries of hypertensive mice occurred (Figure S7B).

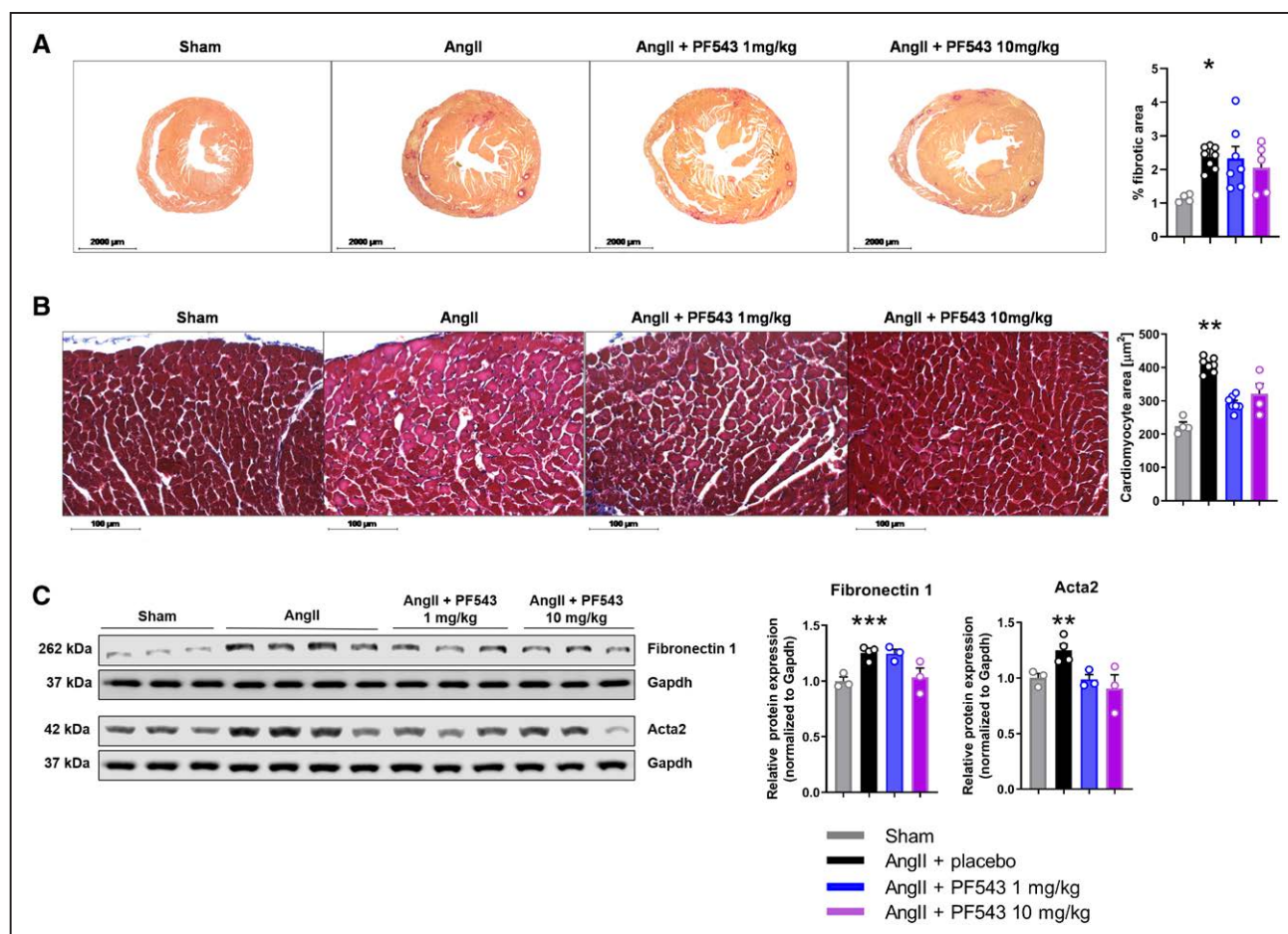


Figure 2. PF543 treatment affects cardiomyocyte hypertrophy and associated Fn1 and Acta2 (α -smooth muscle actin 2) expression with no effects on cardiac fibrosis. **A**, PicroSirius staining of the heart sections used to quantify the percentage of fibrotic area in the cardiac tissue. Percentage of fibrosis is shown relative to the area of heart section. $n=4-8$ /group. **B**, Masson's trichrome staining of the heart sections used to quantify cardiomyocytes area. $n=4-8$ /group. **C**, Protein expression of fibronectin 1 and Acta2 studied by Western blotting in the cardiac tissue of Ang II (angiotensin II)-infused (490 ng/kg per min) mice treated either with PF543 (1 or 10 mg/kg every 2 days) or placebo. Densitometric analysis of protein normalized to Gapdh is shown. $n=3-4$ /group. * $P<0.05$ vs Sham, ** $P<0.05$ vs all other groups, *** $P<0.05$ vs Sham or Ang II+PF543 10 mg/kg groups.

Ang II induced expression of *Sphk1* mRNA in mesenteric arteries as compared with the Sham group; however, PF543 had no further effect on its expression in hypertensive mice (Figure S7C). No differences in *Nos3* mRNA expression were observed between all groups (Figure S7C). Although Ang II infusion did not affect mRNA level of S1P receptors as compared with the Sham group, PF543 triggered an upregulation of *S1pr2* and downregulation of *S1pr3* mRNA in mesenteric arteries of hypertensive mice of relatively little magnitude, that is, increment 1.23 \times and 1.33 \times of *S1pr2* expression in Ang II+PF543 (1 mg/kg per 2 days) and Ang II+PF543 (10 mg/kg per 2 days), respectively, versus Ang II+placebo and 1.31 \times decrement of *S1pr3* mRNA expression in Ang II+PF543 (10 mg/kg per 2 days) versus Ang II+placebo (Figure S7C). As among other S1P receptors S1pr1 plays major role in the regulation of endothelial vasorelaxation, we tested S1pr1 protein level. Results showed no significant difference in S1pr1 protein level between PF543-treated and placebo-treated hypertensive mice (Figure S7D). Moreover, there were no significant differences in arterial expression of genes related to vascular remodeling, that is, *Fnl*, *Colla1*, and *Col3a1* between PF543- and placebo-treated hypertensive animals (Figure S7C).

PF543 and S1P Affect eNOS Phosphorylation In Human Endothelial Cells

PF543 treatment significantly downregulated SPHK1 protein expression by >80% and decreased P-eNOS^{T495} expression in human microvascular endothelial cells (Figure S8A). Additionally, in line with the above results, elevated phosphorylation at the eNOS (T495) site after 24 hours stimulation of endothelial cells with S1P was observed (Figure S8B). No significant changes in phosphorylation of ERK1/2 or PKC were observed after PF543 treatment for 24 hours (Figure S8A). On the contrary, stimulation of endothelial cells with S1P for 24 hours significantly increased P-ERK1/2^{T202/Y204} and decreased PKC phosphorylation level at the S660 site (Figure S8B).

Discussion

In the present study, we demonstrate that pharmacological inhibition of Sphk1 activity modulates vascular function and reduces cardiac hypertrophy induced by Ang II. The latter effect is accompanied by RNA-Seq-quantified downregulation of cytoskeletal, ECM, and TNF α -related genes and may be mediated by the elevation of cardiac sphingosine content and downregulation of S1pr1 and PKC, as well as

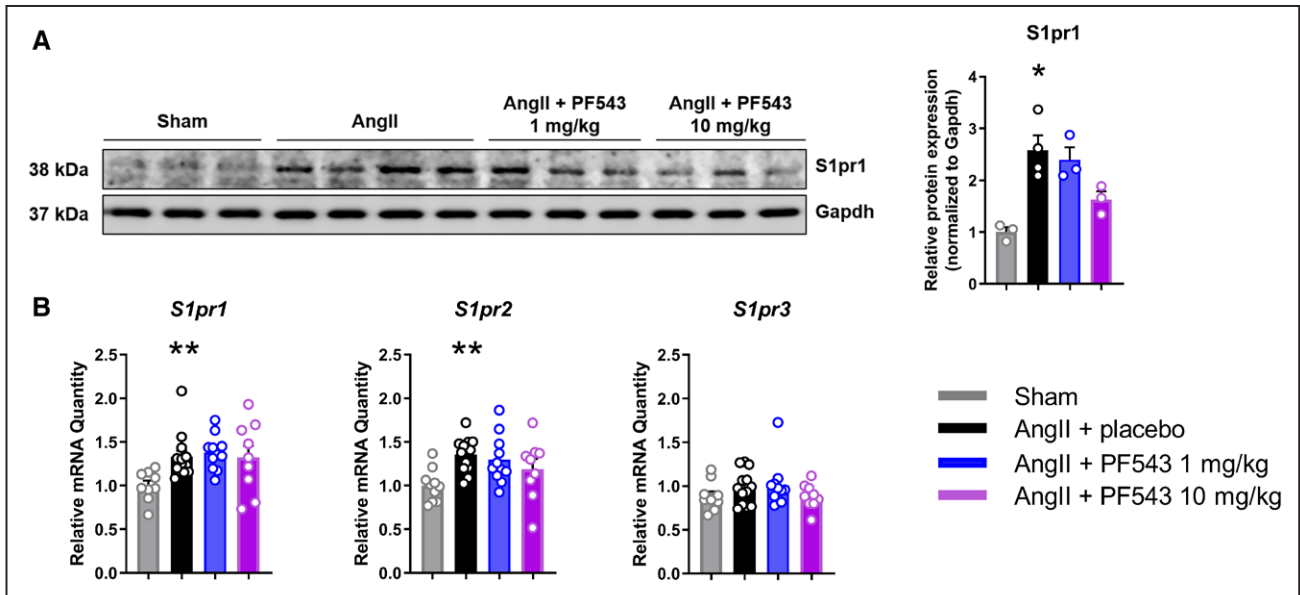


Figure 3. Chronic PF543 treatment downregulates cardiac S1pr1 protein expression in Ang II (angiotensin II)-infused hypertensive mice. **A**, Protein expression of S1pr1 studied by Western blotting in the left ventricular tissue of Sham and Ang II-infused (490 ng/kg per min) mice treated either with PF543 (1 mg/kg or 10 mg/kg every 2 days) or placebo. Densitometric analysis of S1pr1 normalized to Gapdh is shown. $n=3-4$ /group. **B**, Expression of S1pr1, S1pr2, and S1pr3 was quantified using real-time polymerase chain reaction in the hearts of normotensive (Sham) and hypertensive (Ang II-infused) mice treated either with PF543 (1 mg/kg or 10 mg/kg every 2 days) or placebo. $n=9-13$ /group. * $P<0.05$ vs Sham or Ang II+PF543 10 mg/kg groups, ** $P<0.05$ vs Sham group.

pro-hypertrophic markers such as Stat3. This extends our previous, transcriptomic study that identified Sphk1 as a modulator of Ang II-dependent vascular dysfunction and as a potential regulator of cardiac hypertrophy development in response to pressure overload in vivo.⁸

A growing body of literature show that the Sphk1/S1P pathway plays an important role in the cardiovascular system.³³⁻³⁶ MacRitchie et al¹⁹ demonstrated that PF543 degrades Sphk1 in pulmonary vessels in vivo, without effecting Sphk1 expression in the heart, which is in agreement with our results. Another study by Zhang et al²⁰ found that chronic administration of PF543 reduces serum and cardiac levels of S1P in Sham and in post-myocardial infarction mice, which is associated with improved LV ejection fraction. This supports the previous study by Polzin et al³⁷ who demonstrated a negative correlation between LV ejection fraction and plasma S1P levels among patients with ischemic heart disease. We found that PF543 treatment increases sphingosine content and lowers cardiac S1pr1 expression in cardiac tissue, and both phenomena might be caused by various events including Sphk1 inhibition in cardiac tissue or a decrease in serum S1P level, a known consequence of PF543 action in vivo.^{20,38} This extends results of another in vitro study showing a simultaneous decrease in S1P and an increase in sphingosine content in 1483 cells exposed to PF543.³⁹ Moreover, the above results are in line with significant downregulation of sphingosine kinase activity in cardiac tissue after PF543 treatment in vivo observed in the current study.

Regardless of the strategy of administration (acute or chronic injection), PF543 did not affect the level of systolic BP in mice developing or with established hypertension. On the contrary, our findings identified PF543 as a vasorelaxant agent ex vivo and demonstrated a clear linkage between pharmacological administration of PF543 and improvement

of mesenteric artery function in hypertensive mice in vivo. We found a decreased phosphorylation of eNOS at T495, known as an inhibitory for eNOS activation, NO production and endothelium-dependent vasorelaxation,^{40,41} as a possible cause of improved mesenteric artery function after PF543 treatment in hypertensive mice. Furthermore, the effect of PF543 seems to be independent of S1pr1 expression level. We also found significant differences in S1pr2 and S1pr3 mRNA expression after PF543 chronic treatment in mesenteric arteries. Although it is known that signaling mediated by S1pr2/S1pr3 is important for contraction of vascular smooth muscle cells and that S1pr3 positively regulates endothelial vasorelaxation,³⁶ it should be noted that the magnitude of changes in the expression of these receptors observed in our study was relatively small and did not result in changes of arterial contraction or exaggeration of endothelial dysfunction as demonstrated in vascular function studies. The lack of effect of PF543 treatment on vascular contraction may at least partially explain no significant effects observed with regards to BP level during hypertension development.

Among kinases targeting eNOS at T495 such as PKC,⁴² Rock1,⁴³ and ERK1/2,^{41,44} only expression of the phosphorylated ERK1/2 was attenuated by chronic PF543 treatment. Moreover, downregulation in T495 eNOS phosphorylation in response to PF543, which mimics inhibition of autocrine S1P signaling, was observed also in vitro in human primary microvascular endothelial cells. Stimulation of these cells with S1P, which mimics upregulation of endocrine S1P signaling in vivo, evoked the opposite effect and hence confirmed that S1P regulates eNOS activity by its phosphorylation at T495. While PF543 did not affect ERK1/2 or PKC phosphorylation level, S1P stimulation significantly upregulated P-ERK1/2, which confirms previous findings by Igarashi et al.⁴⁵ In summary,

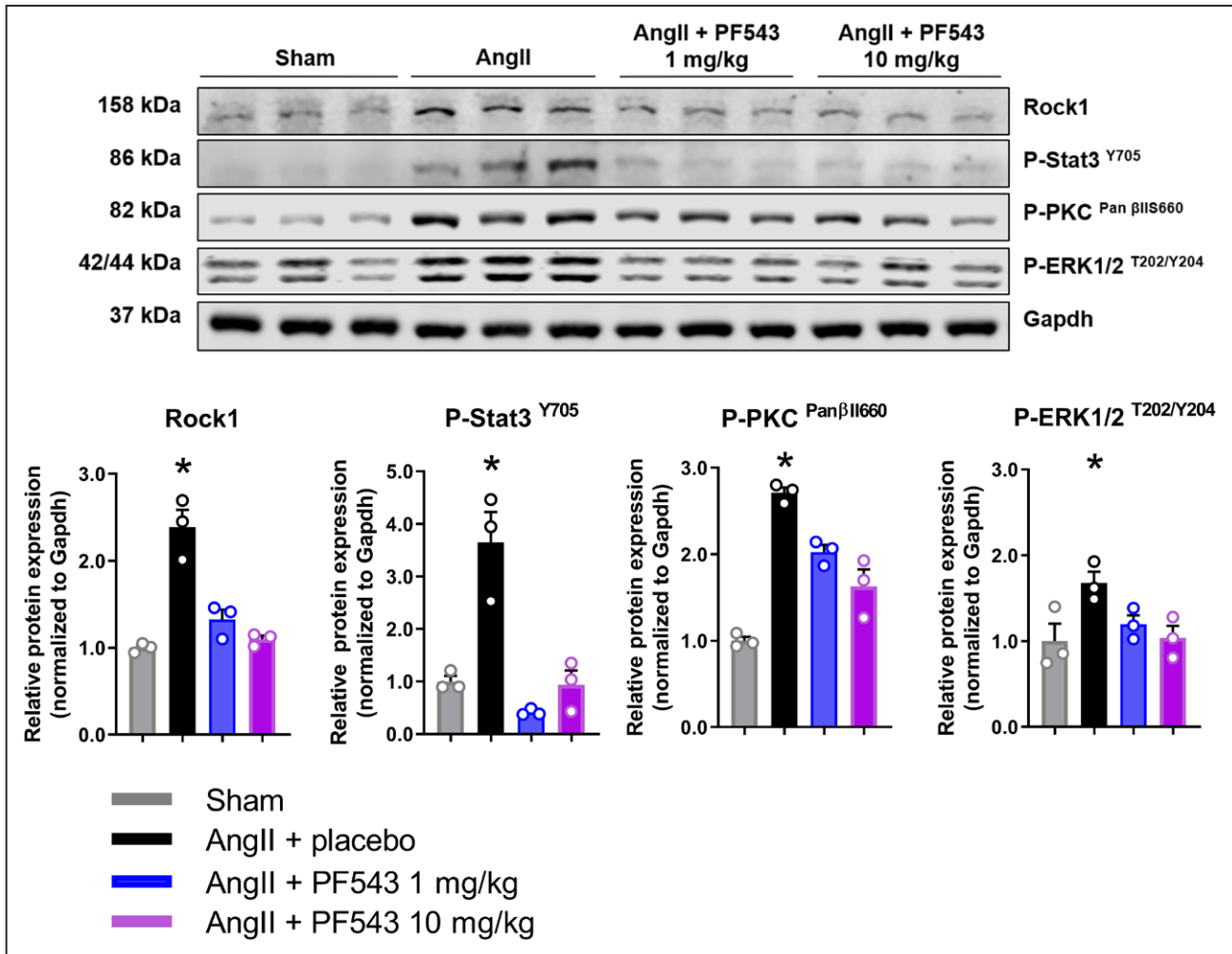


Figure 4. Chronic PF543 treatment downregulates Rock1 (Rho-associated coiled-coil containing protein kinase 1), P-Stat3 (signal transducer and activator of transcription 3), P-PKC (protein kinase C), and P-ERK1/2 (extracellular signal-regulated kinases 1/2) in cardiac tissue of Ang II (angiotensin II)-infused hypertensive mice. Protein expression of Rock1, p-Stat3^{Y705}, P-PKC^{PanβII660}, P-ERK1/2^{T202/Y204} and Gapdh studied by Western blotting in the hearts of Sham or Ang II-infused (490 ng/kg per min) mice treated either with PF543 (1 mg/kg or 10 mg/kg every 2 days) or placebo. Densitometric analysis of proteins normalized to Gapdh is shown. n=3/group.*P<0.05 vs all other groups.

these findings show that a relative improvement of endothelial function caused by PF543 is not sufficient to compensate for other Ang II-stimulated mechanisms contributing to the development of experimental hypertension.

A 2-week infusion of Ang II resulted in the development of cardiac hypertrophy. Ang II triggered an upregulation of fibronectin 1, an important stimulator of cardiomyocyte hypertrophic growth,⁴⁶ which action is transduced by membrane heterodimeric integrin receptors composed of an α and β chain. Pharmacological inhibition of Sphk1 by PF543 prevented Ang II-induced transcript upregulation of *fibronectin 1*, integrins (*Itga5* and *Itgb3*), *Ctgf*, *Tgf- β 1*, and fetal hypertrophy markers *Nppb* and *Myh7* in hypertensive animals. Since PF543 significantly reduced cardiomyocyte size in hypertensive animals, we conclude that PF543 may serve as a potential antihypertrophic agent due to its effect on S1P/sphingosine balance in cardiac tissue and serum,^{20,38} and its action may be mediated by both S1P-receptor dependent and independent pathways.

Spatial distribution of S1P receptors in the heart is an important determinant of specific S1P action in different cardiac tissue compartments. While cardiac fibroblasts express

mainly S1pr3, cardiomyocytes are characterized by abundant S1pr1 expression.¹⁴ Our results indicate that reduced cardiac hypertrophy due to PF543 treatment may be mediated, at least partially, by a downregulation of S1pr1 expression. This result ties well with previous studies where S1pr1 antibody treatment prevented S1P-induced increase in the size of rat neonatal cardiomyocytes,¹⁶ and genetic overexpression of S1pr1 in myofibroblasts exaggerated cardiac hypertrophy in an Ang II-dependent manner in vivo.¹⁸ Since we did not observe significant effects of PF543 on cardiac fibrosis in hypertensive mice, as assessed by collagen-staining method, it can be concluded that pharmacological inhibition of Sphk1 affects Ang II-dependent cardiac hypertrophy rather than fibroblast-mediated deposition of collagen. This is in line with our previous study that investigated the *Sphk1* knockout mouse for these effects.⁸

To further investigate the mechanism of attenuation of Ang II-dependent cardiac hypertrophy by PF543, we checked the activation status of various signaling molecules. A positive correlation of Ang II infusion with elevated activity of signaling molecules such as Rock1, Stat3, PKC,

Akt, and ERK1/2 in the hypertrophic heart was observed. Crucially, pharmacological inhibition of Sphk1 with PF543 remarkably downregulated Rock1, as well as decreased the activation status of Stat3, PKC, and ERK1/2. Rock1 is an important positive regulator of cardiomyocyte apoptosis and cardiac fibrosis; thus it is considered as potential therapeutic target for limiting the progression to heart failure.⁴⁷ Stat3 is a transcription factor regulating the expression of cardio-protective genes associated with the adaptation of the heart to stress conditions.⁴⁸ Activation of Stat3 in pressure-overloaded cardiac myocytes is primarily mediated by the auto-crine/paracrine release of IL-6 and TNF- α , and to a lesser extent, by the local production of Ang II.⁴⁹ Importantly, it was also revealed that activation of Stat3 in neonatal rat ventricular cardiomyocytes is induced by increasing the dose of S1P, and this effect is mediated by upregulation of P-ERK1/2. Additionally, S1P-mediated ERK1/2 and Stat3 activation was partially reversed by treatment with an inhibitor of Rock (Y27632), which points to Rock as a potential mediator in S1P signaling in cardiomyocytes.⁵⁰ Moreover, several studies demonstrated that both canonical (α , β) and novel isoforms (δ , ϵ) of PKC regulate pathological cardiac remodeling.⁵¹ Importantly, sphingosine is a known PKC inhibitor, and higher sphingosine content in LV tissue induced by PF543 treatment may explain PF543-induced downregulation of cardiac PKC phosphorylation.

As S1P in the heart has been considered cardioprotective,¹⁴ questions arise as to whether reduced cardiac hypertrophy after chronic PF543 treatment does not result from enhanced cell death. However, RNA-Seq analysis of cardiac transcriptome, as well as determination of the level of markers and mediators of cardiac apoptosis and myocyte survival (ie, PARP, P-Akt, and P-GSK3 β) demonstrated no significant differences between placebo and PF543-treated mice.

Inclusion of exclusively male animals may be considered as a limitation of our study. This was associated with the fact that Ang II infusion is best characterized as model of hypertension in male mice.⁵² Studies demonstrated that Ang II induces considerably greater blood pressure increase in male than in female subjects, and that sex hormones are important mediators of hypertension pathogenesis.⁵³ Moreover, there is a higher plasma S1P level in woman, as well as in female mice than in males or male mice, respectively.⁵⁴ Considering the fact that some of the mechanisms investigated in the current study may be altered by the above mentioned sex-mediated differences, it remains to be established whether similar, cardiac effects of PF543 treatment can be observed in female mice that are at least partially protected against Ang II-induced hypertension due to various hormone- and immunology-related factors.⁵⁵ Future studies are needed to characterize sex-dependent differences in S1P biology in hypertension.

Perspectives

PF543 has an anti-hypertrophic potential as it partially protects against pathological changes that occur in the heart during Ang II-dependent hypertension in mice. Its action may be mediated by specific downregulation of S1P type 1 receptor expression, as well as by sphingosine accumulation in the cardiac tissue as a result of Sphk1 inhibition. PF543 triggers

downregulation of important cardiac hypertrophy-related pathways mediated by Rock1, Stat3, PKC, and ERK1/2. Furthermore, PF543 partially inhibits changes in the expression of actin-, TNF α -, and tissue remodeling-associated genes that are characteristic for the hypertrophic heart. Thus, SPHK1 inhibition might be a pharmacological strategy to prevent cardiac hypertrophy in patients with newly diagnosed hypertension. Chronic rather than acute effects of SPHK1 inhibition are more likely.

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Disclosures

None.

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Novelty and Significance

What Is New?

- Pharmacological inhibition of Sphk1 (sphingosine kinase 1) with PF543 reduces endothelial dysfunction in the arteries from Ang II (angiotensin II)-infused, hypertensive mice as compared with placebo-treated controls with no effect on systolic blood pressure level. This effect is associated with a downregulation of ERK1/2 (extracellular signal-regulated kinases 1/2) signaling and a reduction of inhibitory eNOS (endothelial nitric oxide synthase) phosphorylation at T495.
- PF543 chronic treatment reduces cardiac hypertrophy in Ang II-infused, hypertensive mice. Mechanisms of action may be partially mediated by the downregulation of S1pr1, Rock1 (Rho-associated coiled-coil containing protein kinase 1), Stat3 (signal transducer and activator of transcription 3), PKC (protein kinase C), and ERK1/2 signaling.

- PF543 chronic treatment reduces expression of cardiac actin-, TNF- α (tumor necrosis factor- α)-, and tissue remodeling-associated genes that are upregulated by Ang II.

What Is Relevant

- Our results demonstrate novel, pharmacological effects of Sphk1 inhibition in the cardiovascular system of hypertensive mice.

Summary

PF543 has anti-hypertrophic properties as it partially protects against pathological hypertrophic changes that occur in the heart during experimental Ang II-dependent hypertension.