Vascular transcriptome profiling identifies *Sphingosine kinase 1* as a modulator of angiotensin II-induced vascular dysfunction

Mateusz Siedlinski¹, Ryszard Nosalski¹, Ryszard Nosalski¹, Piotr Szczepaniak¹, Agnieszka H. Ludwig-Gałęzowska³, Tomasz Mikolażycki¹, Magdalena Filip¹, Grzegorz Osmenda¹, Grzegorz Wilk¹, Michał Nowak¹, Paweł Wolkow³ & Tomasz J. Guzik¹, ²

Vascular dysfunction is an important phenomenon in hypertension. We hypothesized that angiotensin II (AngII) affects transcriptome in the vasculature in a region-specific manner, which may help to identify genes related to vascular dysfunction in AngII-induced hypertension. Mesenteric artery and aortic transcriptome was profiled using Illumina WG-6v2.0 chip in control and AngII infused (490 ng/kg/min) hypertensive mice. Gene set enrichment and leading edge analyses identified *Sphingosine kinase 1* (*Sphk1*) in the highest number of pathways affected by AngII. Sphk1 mRNA, protein and activity were up-regulated in the hypertensive vasculature. Chronic sphingosine-1-phosphate (S1P) infusion resulted in a development of significantly increased vasoconstriction and endothelial dysfunction. AngII-induced hypertension was blunted in *Sphk1*⁻/⁻ mice (systolic BP 167 ± 4.2 vs. 180 ± 3.3 mmHg, p < 0.05), which was associated with decreased aortic and mesenteric vasoconstriction in hypertensive *Sphk1*⁻/⁻ mice. Pharmacological inhibition of S1P synthesis reduced vasoconstriction of mesenteric arteries. While Sphk1 is important in mediating vasoconstriction in hypertension, *Sphk1*⁻/⁻ mice were characterized by enhanced endothelial dysfunction, suggesting a local protective role of Sphk1 in the endothelium. S1P serum level in humans was correlated with endothelial function (arterial tonometry). Thus, vascular transcriptome analysis shows that S1P pathway is critical in the regulation of vascular function in AngII-induced hypertension, although *Sphk1* may have opposing roles in the regulation of vasoconstriction and endothelium-dependent vasorelaxation.

Hypertension is a pathogenetically complex disorder affecting more than 1 billion people worldwide. Etiology of the disease remains unknown in the majority of adult patients. It is widely acknowledged that function of the vascular system, kidney and sympathetic nervous system is critical for regulating blood pressure (BP) levels. Since the existence of genetic predisposition to develop abnormally high BP is well known, significant efforts have been made in order to identify particular genes affecting hypertension using genome-wide association studies (GWAS). However, clinical translation of such studies is in the early phase and requires detailed characterization of the effect of certain genes and molecular pathways on the function of key organs involved in BP regulation. High throughput techniques significantly contributed to unraveling mechanisms of complex disorders and phenotypes such as type II diabetes, chronic obstructive pulmonary disease or hypertension and BP regulation in humans. All the above studies focused on the association between genetic polymorphisms and disease status using Mendelian randomization concept allowing establishing causal relationships. Complexity of the pathogenesis of hypertension makes uncovering key drivers of the disease difficult in humans. Models of the disease such as angiotensin II (Ang II) infusion or DOCA salt hypertension, represent most common causes of primary hypertension in humans and allowed to unravel the important mechanisms of hypertension and associated...
Systemic vascular resistance is critical for the physiological regulation of blood pressure. At the same time, hypertension promotes development of vascular dysfunction and atherosclerosis in the larger vessels. Importantly, while vascular oxidative stress and inflammation have been clearly linked to hypertension and its consequences[8,9], the effects of chronic Ang II infusion on distinct vascular beds is yet to be addressed in a systematic manner.

Vascular dysfunction may have divergent mechanisms and consequences in various vascular beds. In the aorta, it may represent vascular target organ damage but may also contribute to vascular stiffening important for hypertension development. In mesenteric arteries, which represent typical resistance vessels, increased vascular tone may directly translate into increased blood pressure.

Investigation of transcriptome creates an opportunity for gaining a unique insight into potential vascular mechanisms of Ang II-dependent hypertension. Transcriptome profiling studies ex vivo have so far focused on kidneys following 2 days Ang II infusion[10], heart in the model of Ang II–induced cardiac fibrosis[11,12] or abdominal aortic aneurysm in ApoE knockout mice[13]. Despite the difficulties with obtaining cell-type specific and homogenous RNA pool, all the above studies clearly showed that Ang II displays systemic effects on transcriptome and important information on the disease process studied could be obtained using this high-throughput technique.

The aim of current study was to profile the transcriptome of key vessels of normotensive and hypertensive mice using Ang II-induced hypertension model and further to identify genes and biological pathways that may causally affect development of hypertension in vivo. Both thoracic and abdominal regions of the aorta, selected in the current study, are characterized by hypertrophy following Ang II treatment in vivo[14], yet they show different expression pattern of Ang II receptor subtypes AT1a and AT1b[15,16]. This may confer differential roles of these areas of the aorta in hypertension[15,16], thus investigation and characterization of these two regions is a key to the understanding mechanism of Ang II action in an in vivo model. Superior mesenteric vascular bed, which is another part of the vasculature selected for transcriptome profiling, is a part of the vascular resistance system, critical for blood flow regulation[17,18]. Since mesenteric arteries express AT1 receptors and are susceptible to Ang II-mediated dysfunction in vivo[19,20], investigation of similarities and differences in gene expression in these three areas of vasculature may provide important molecular information on specific changes occurring during Ang II-mediated hypertension in vivo.

Results
Transcriptome analysis. Approximately 16,000 (out of 45,281) RNA transcripts were detected (detection p value < 0.01) in studied vascular tissues i.e. thoracic aorta, abdominal aorta and mesenteric arteries. Principal component analysis comprising RNA transcripts detected in all tissues provided a clear separation of samples according to tissue type and treatment status i.e. hypertensive vs. normotensive (Fig. 1a). Out of all transcripts expressed, 21.5% were differentially expressed (FDR corrected p < 0.05) in thoracic aorta between hypertensive and normotensive mice, while such proportion was lower for abdominal aorta (0.3%) or mesenteric arteries.
Table 1. Ten genes present in the highest number of gene sets most significantly enriched in hypertension in thoracic aorta and mesenteric arteries and their expression fold change in hypertensive mice as compared to normotensive mice. log2FC = logarithm of Fold Change (hypertensive vs. normotensive mice).

| Gene       | Number of gene sets (out of 68) | log2FC  | Expression change–FDR adj. p value | Gene       | Number of gene sets (out of 36) | log2FC  | Expression change–FDR adj. p value |
|------------|---------------------------------|---------|-----------------------------------|------------|---------------------------------|---------|-----------------------------------|
| Cdc13      | 30                              | 0.595   | 0.0005                            | Igf1p5     | 9                               | 0.17    | 0.11                              |
| Sphk1      | 29                              | 1.223   | 0.0003                            | Eif5a      | 9                               | 0.15    | 0.24                              |
| Sod1       | 29                              | 0.191   | 0.0391                            | Inhba      | 8                               | 0.19    | 0.39                              |
| Tfy1       | 28                              | 0.794   | 0.0013                            | Mast2      | 8                               | 0.17    | 0.87                              |
| Tgfb1      | 22                              | 0.284   | 0.0248                            | Cdc25      | 7                               | 0.06    | 0.49                              |
| Nmc2       | 21                              | 0.283   | 0.0092                            | Ccdd88a    | 7                               | 0.10    | 0.28                              |
| Col1a3     | 21                              | 0.245   | 0.0278                            | Sphk1      | 7                               | 0.40    | 0.04                              |
| Vegfa      | 20                              | 0.330   | 0.0594                            | Apbb1      | 7                               | 0.17    | 0.23                              |
| Angpt1     | 19                              | 0.402   | 0.0426                            | Anacp5     | 7                               | 0.18    | 0.17                              |
| Huatip2    | 18                              | 0.835   | 0.0002                            | Ppp5c      | 6                               | 0.09    | 0.41                              |

(1.4%). Among differentially expressed genes 46%, 91% and 67% were overexpressed in hypertensive as compared to normotensive mice in the thoracic aorta, abdominal aorta and mesenteric arteries respectively.

Among top 200 RNA transcripts associated with Ang II-induced hypertension in either of the examined tissues, 3 genes were previously associated with BP in GWA cohort studies. Expression of both subunits of the soluble guanylate cyclase (Gucy1a3 and Gucy1b3) was significantly downregulated in both aortic compartments, but not in mesenteric arteries, in hypertensive mice as compared to control, while Cyclin M2 (Cmm2) expression was significantly upregulated in mesenteric arteries of hypertensive mice as compared to control. None of the above genes showed a similar pattern of expression change following Ang II infusion in all vascular compartments studied.

Gene set enrichment analysis identified 68 gene sets significantly enriched in hypertension in thoracic aorta (see Supplementary Table 1 for top 20 sets). Subsequent leading edge analysis performed on these sets identified genes present in the highest number of pathways upregulated in Ang II-induced hypertension (Table 1). Among them, only Sphk1 showed more than a 2-fold induction of expression in thoracic aorta of hypertensive mice as compared to control (Table 1).

Similar gene set enrichment analysis identified no pathways significantly enriched in hypertension in mesenteric arteries at FDR p value < 0.05. Therefore, leading edge analysis was based on 36 gene sets (see Supplementary Table 1 for top 20 sets) with normalized enrichment score > 1.4 (nominal p value < 0.05) in mesenteric arteries. This analysis also identified Sphk1 as one of the members of the highest number of gene sets enriched in mesenteric arteries following Ang II infusion (Table 1). Importantly, Sphk1 gene was also among the 3 genes that changed in response to Ang II in all three vascular beds and among genes affected by Ang II infusion globally i.e. irrespectively of tissue type (Fig. 1b,c).

**Sphk1 expression and activity in vasculature.** Subsequent real-time PCR confirmed the above findings and showed a significant induction of Sphk1 mRNA expression in all vascular compartments studied, while no significant effects of Ang II on Sphk2 expression in vivo were observed (Fig. 2a). These increases translated into a significant increase in protein levels as exemplified by Western blotting (Fig. 2b and Supplementary Figure 1a,b). We observed a significant, yet modest, 1.8x increase in Sphk1 protein expression and activity in vasculature.

Moreover, induction of vascular Sphk1 mRNA by Ang II infusion persisted when blood pressure raise was reduced by 76% by administration of hydralazine (Fig. 2c), indicating that increases observed were not a simple result of blood pressure raise but were likely caused by direct actions of Ang II in vivo.

The above observations were accompanied by a significant, 2.8x increase in sphingosine kinase activity as well as moderate increment in sphingosine-1-phosphate (S1P) concentration (Fig. 2d) in aortic homogenates of AngII-infused mice as compared to Sham control respectively.

An independent experiment focusing on different layers of thoracic aorta showed that Ang II effects on Sphk1 expression in vivo were more pronounced in the media layer (Induction Fold (IF) = 14.2, p < 0.05) as compared to adventitia layer (IF = 3.6, p = 0.077) or endothelial cell -enriched fraction representing intima (IF = 4.5, p < 0.05, Fig. 2e). In baseline conditions expression of Sphk1 was 3.2x and 18.1x higher in the media layer as compared to adventitia or intima respectively (Fig. 2e).

**Effects of chronic S1P infusion on vascular function in vivo.** AngII-induced hypertension was accompanied by a significant increase in plasma S1P level in wild-type (WT) mice (Fig. 3a,b). Therefore we studied the effects of chronic S1P delivery in normotensive mice in vivo. Chronic S1P infusion resulted in a 35% increment in plasma S1P level that was accompanied by a moderate, yet not significant increase in SBP by 10 mmHg in vivo (p = 0.35). S1P infusion resulted in significantly elevated degree of endothelial dysfunction and vascular contractility in mesenteric arteries (Supplementary Figure 2a–c).
Sphk1 deficiency and hypertension development in vivo. There was no significant difference in baseline blood pressure between Sphk1−/− and WT mice (Fig. 3a). However, upon infusion of Ang II, Sphk1−/− mice developed significantly less severe Ang II-induced hypertension in vivo as compared to WT mice (Fig. 3a). Importantly, no significant difference in heart rate was observed at both baseline conditions and upon Ang II infusion between WT and Sphk1−/− mice (data not shown). Following 14-days Ang II infusion period Sphk1−/− mice developed less severe cardiac hypertrophy as compared to WT mice (Heart to body weight ratio for Ang II vs. Sham groups: 5.9 ± 0.22 mg/g vs. 5.5 ± 0.15 mg/g (p = 0.15) in Sphk1−/− mice and 6.8 ± 0.21 mg/g vs. 5.8 ± 0.21 mg/g (p < 0.05) in WT mice). This was confirmed by an analysis of cardiomyocyte size (Supplementary Figure 3a,b). Further analyses revealed that effects of Ang II on fibrotic changes in the heart as well as IMT of cardiac arteries were similar in WT and Sphk1−/− mice (Supplementary Figure 3c,d).

Effects of Ang II and Sphk1 deficiency on S1P level and expression of S1P receptors S1pr1 and S1pr2. Sphk1−/− mice were characterized by 2–3x lower S1P plasma level as compared to WT mice (Fig. 3b) and approximately 30% ± 3% (p < 0.05) lower Sphk activity in the aorta. No significant compensatory effect of Sphk1 deletion on Sphk2 expression in vasculature was observed (data not shown). No significant effects of Ang II infusion on Sphk1−/− mice developed less severe cardiac hypertrophy as compared to WT mice (Heart to body weight ratio for Ang II vs. Sham groups: 5.9 ± 0.22 mg/g vs. 5.5 ± 0.15 mg/g (p = 0.15) in Sphk1−/− mice and 6.8 ± 0.21 mg/g vs. 5.8 ± 0.21 mg/g (p < 0.05) in WT mice). This was confirmed by an analysis of cardiomyocyte size (Supplementary Figure 3a,b). Further analyses revealed that effects of Ang II on fibrotic changes in the heart as well as IMT of cardiac arteries were similar in WT and Sphk1−/− mice (Supplementary Figure 3c,d).

Effects of Sphk1 deficiency and pharmacological modulation on vascular contractility. Pre-incubation of WT thoracic aorta with S1P significantly increased vasoconstriction of aortic rings in response
to phenylephrine (Phe) (Fig. 4a). This effect was also observed in mesenteric arteries and was also evident in vessels isolated from Sphk1−/− mice (data not shown). On the contrary, Sphk1 knockout led to a blunted increases in aortic and mesenteric arteries contractility to Phe and noradrenaline (respectively) in Ang II – hypertensive mice (Fig. 4b,c). In order to address this further, we studied the effects of S1P pathway modulators on vascular function ex vivo. Pre-incubation of mesenteric arteries with either Sphk1 inhibitor PF-543 or S1PR2 antagonist (JTE-013), significantly reduced Ang II-induced contraction ex vivo (Fig. 4d). Further tests performed on aortic rings showed that these inhibitory effects of JTE-013 on vessel contraction were dose-dependent and significant at a concentration of 100 nM (Supplementary Figure 4a).

Effects of Sphk1 deficiency on endothelial function and vascular superoxide anion production. Lack of Sphk1 was associated with a significant reduction of vasorelaxation in response to acetylcholine (Ach) in aortas of Ang II-infused mice (Fig. 5a) when compared to WT aortas from Ang II-infused mice. A similar trend was observed in mesenteric arteries (Fig. 5b). No differences in sodium nitroprusside (SNP)-induced vasodilation between WT and Sphk1−/− mice were observed (Supplementary Figure 4b,c). While Sphk1−/− was linked to concomitant enhancement of endothelial dysfunction with opposing decreased vascular contractility and moderately reduced blood pressure, it had no significant effect on intima media – thickness hypertrophy in Ang II-induced hypertension (Fig. 5c,d).

Significantly elevated CYBB (encoding Nox2 NADPH oxidase) expression in hypertensive Sphk1−/− mice as compared to hypertensive WT mice, both in the aorta and mesenteric arteries, was observed (Supplementary Figure 5a). No significant changes in Nox1 or Nox4 expression between Sphk1−/− and WT mice were observed (data not shown). AngII infusion significantly elevated production of superoxide anion in both Sphk1−/− and WT mice. However, this increment was significantly higher in Sphk1−/− mice as compared to WT mice (Supplementary Figure 5b). Significant experiments performed on aortic homogenates using fluorescent dihydroethidium (DHE) marker, showed a similar trend of association which was not significant (p = 0.22, for comparison of hypertensive Sphk1−/− vs WT mice).

In summary, results obtained using Sphk1−/− model and acute pharmacological modulation indicate that S1P generated via Sphk1 pathway exerts pro-contractile effects within vascular smooth muscle cells (VSMC) and an opposing protective role on the level of endothelium function.

Serum S1P levels and vascular function in humans. Serum S1P level significantly correlated with impaired endothelial function (lower ln (Reactive Hyperemia index-RHI)) and higher aortic systolic pressure (ASP) levels, in patients with cardiovascular risk, assessed by EndoPAT and SphygmoCor respectively. At the
same time, serum S1P was not significantly associated with age, sex or smoking status. No significant correlation between S1P and absolute office SBP, DBP, IMT, pulse wave velocity (PWV) or augmentation index (AIx) was observed (Table 2). Analysis of dichotomized variables showed that subjects with abnormal lnRHI (n = 20) or AIx (n = 19) level had significantly higher S1P level (respectively median of 22.3 (IQR:18.6–25.2) μM and 22.3 (IQR:16.8–25.5) μM) as compared to the rest of subjects (respectively median of 19.4 (IQR:16.2–22.6) μM and 19.5 (IQR:16.2–22.8) μM). No such association existed for dichotomized PWV level, confirming links of S1P to endothelial function rather than vascular stiffness.

Discussion
Bioinformatical analysis of data obtained via transcriptome profiling, employed in the current study, identified Sphk1 as one of the key modulators of Ang II-dependent vascular dysfunction in vivo. While numerous genes are altered during the development of Ang II-dependent hypertension, only Sphk1, Srpx and Mfap4 were common for different vascular areas studied. Sphk1 was identified as a member of numerous key biological pathways significantly altered in hypertension and its expression was induced in major arteries following Ang II stimulation in vivo. Therefore we postulated that Sphk1 would play a critical role in the regulation of vascular function, BP increase, and consequently cardiac hypertrophy in hypertension. Indeed, using Sphk1−/− mice we observed that Sphk1 is causally linked with BP elevation upon chronic Ang II infusion. Ex vivo and in vivo experiments showed that diminished vascular contractility is a possible mechanism contributing to a partial protection from Ang II-induced hypertension in mice lacking Sphk1. Surprisingly, the reduction of BP increase was only moderate and was not associated with a significant reduction of vascular dysfunction characterized by intima-media hyper trophy. Further studies have identified that in opposition to overall protection from hypertension and decreased vascular contractility, endothelial dysfunction was significantly aggravated in Sphk1−/− mice. These results clearly point to the critical but very complex role of Sphk1 in the pathogenesis of Ang II-dependent hypertension and vascular dysfunction.

S1P is a lipid signaling molecule with numerous functions in vascular homeostasis, including signal transduction, mediating proliferation and migration of endothelial cells (EC), VSMCs and cardiac myocytes with a possibly clinically relevant role in human diseases such as pulmonary hypertension or abdominal aortic aneurysm. It acts via 5 known receptors and S1PR1, S1PR2 and S1PR3 are predominant in the cardiovascular system.
Sphk1 plays a critical role in the generation of S1P and Sphk1−/− mice are characterized by a systemic reduction in Sphk1 activity and depletion of S1P24,25. Identification of Sphk1 as a key member of numerous pathways altered in hypertension, caused by chronic Ang II infusion, is important, as it is in line with the previous report by Wilson and colleagues who found Sphk1−/− mice to be protected against 2-weeks model of Ang II-induced hypertension26, which was not confirmed in the acute model of Ang II injection27. In the current study, we found a somewhat smaller reduction in BP raise in Sphk1−/− mice following chronic Ang II infusion as compared to the previous study26, which could be caused by a slightly lower dose of Ang II-infused. We believe that a moderate reduction in BP raise in Sphk1−/− mice observed by us is caused by opposing effects of S1P in the VSMCs and ECs. Previous studies have identified possible mechanisms of pro-hypertensive effects of Sphk1 including intracellular role of S1P in calcium entry via targeting store operated calcium channels26. Our data may support and extend these findings, since we observed both, in vivo and ex vivo, a lower

![Figure 5. Effects of Sphk1 deletion on endothelial function and intima-media thickness. (a,b) Tissue organ bath was performed using 2 mm aortic rings (a) or 2nd order mesenteric arteries (b) from WT and Sphk1−/− mice with an assessment of relaxation in response to increasing concentrations of acetylcholine. Vessels were precontracted using concentration of vasoconstricting agent (PGF2α or noradrenaline) required to achieve 90% of maximal contraction as determined by testing increasing doses of certain vasoconstrictor, n = 6/group; (c,d) Masson's trichrome staining of representative aortas of WT and Sphk1−/− mice (c) and comparison of mean IMT of all groups studied (d), n = 5 for Sham groups, n = 7 for AngII groups; *p < 0.05. WT = wild type; Ach = acetylcholine; SNP = sodium nitroprusside, [M] = molar concentration.]

|                      | Spearman's R coefficient | p-value |
|----------------------|--------------------------|---------|
| LnRHI                | −0.221                   | 0.046   |
| ASP                  | 0.214                    | 0.049   |
| DBP                  | 0.204                    | 0.066   |
| SBP                  | 0.107                    | 0.337   |
| Average IMT          | −0.044                   | 0.696   |
| PWV                  | −0.038                   | 0.733   |
| Alx                  | 0.017                    | 0.882   |

Table 2. Serum S1P level and its correlations with various parameters of vascular function. RHI = Reactive Hyperemia Index, Alx = Augmentation Index, ASP = Aortic Systolic Pressure, S1P = sphingosine-1-phosphate, IMT = intima-media thickness, PWV = pulse wave velocity.
contraction of vessels with lower activity of Sphk1 in response to Phe as compared to vessels with normal Sphk1 activity. This indeed suggests Sphk1-mediated Ca\(^{2+}\) handling, since receptor-mediated membrane depolarization includes engagement of voltage-dependent calcium channels and the previous study demonstrated that S1P can activate CREB transcription factor via indirect Ca\(^{2+}\) influx due to inhibition of voltage-gated K\(^{+}\) channels in VSMCs. We attempted to further identify downstream molecular targets responsible for a lower contractile response in vessels with diminished Sphk1 activity. We found that blocking S1PR2 receptor lowers both aortic and mesenteric arteries contraction in response to various stimuli. Since there is an ongoing debate concerning the specificity of antagonizing S1PR2 by JTE-013, which may have off-site effects especially in micromolar concentration, we addressed this issue by testing different doses of JTE-013 and found that nanomolar concentrations of this antagonist reduce vascular contraction as well. However this effect likely disappears below 10 nM concentration of JTE-013, thus there is a need to develop a specific S1PR2 antagonist, which would facilitate studies on a role of this receptor in vessel contraction. The potential role of S1pr2 in AngII-mediated impairment of vascular function is further supported by the observed induction of expression of this receptor in the aorta of WT Ang II-infused mice, which consequently may partially mediate Ang II effects in vivo. Interestingly, S1pr2\(^{−/−}\) mice have similar BP as compared to WT controls in basal conditions. However, contractile responses of vessels of S1pr2\(^{−/−}\) mice to KCl or Phe are impaired ex vivo and in vivo stimulation of S1pr2\(^{−/−}\) mice with Phe unravels lower mesenteric and renal vascular resistance and a lower mean arterial pressure as compared to WT animals.

However, our study reveals the complexity of the effects of Sphk1 on the regulation of endothelial function as well. While in VSMCs S1P-dependent calcium alterations may regulate contractility via store-operated Ca\(^{2+}\) channels and S1pr2, in endothelium its effects may be completely different. Our findings in in vivo hypertension model suggest that S1P may be an important marker of endothelial dysfunction. Experiments using Sphk1\(^{−/−}\) mice suggest S1P level lack of Sphk1 and low plasma S1P level associates with impaired endothelial function. On the other hand, hypertensive WT mice display elevated S1P level, thus we tested in vivo effects of chronic S1P delivery. We found that elevation of S1P plasma level associates with significant impairment of endothelial function as well as with increased vessels contractility. It is of future interest to address exact mechanisms of this finding, which, among others, may include abnormal activation of S1prs in vascular media responsible for vessel contraction and internalization or degradation of S1pr1 in endothelium due to high S1P level. It has been shown that genetic or pharmacological inhibition of S1pr1 or S1pr3 does not impair Ach-induced relaxation. On the other hand, agonists of S1prs such as Fingolimod or S1P may display vasorelaxant properties only when S1pr1 and S1pr3 are active. Previous studies showed that sphingosine kinase promotes phosphorylation and activation of endothelial nitric oxide synthase especially via S1pr1 receptor, and associated MAPK and PI3K/Akt pathways, in order to counteract Ang II effects and to mediate various processes such as angiogenesis. Moreover, stimulation of S1pr1 receptor with specific S1pr1 agonist SEW2871 in vivo may lower BP in Ang II-induced hypertension.

As recently shown by Cantalupo and colleagues, endothelial sphingolipid metabolism remains under control of Nogo-B transcription factor, which inhibits expression of serine palmitoyltransferase that participates in the synthesis of sphingolipids in vivo. Mice lacking Nogo-B in endothelium were protected against Ang II-induced hypertension and endothelial dysfunction, which was not the case for mice lacking Nogo-B in VSMC that were only partially protected against Ang II-induced hypertension. The latter effect could be explained by a reduced myogenic tone in VSMC-Nogo-B deficient mice. These various effects of inhibition of sphingolipid synthesis on vascular contractility or endothelial function can possibly explain relatively moderate, yet significant, effect of Sphk1 deletion on blood pressure raise in Ang II-induced hypertension in the current study, which, in our opinion, deserves further investigation. Creation of VSMC and EC-specific Sphk1\(^{−/−}\) mice would be of high value to dissect the role of this kinase in vascular function in vivo.

Recent work of Tolle and co-workers demonstrated that S1P plays a role in activation of human eNOS since both S1PR1 and S1PR3 were essential for eNOS activation in human endothelial HUVEC cells. However, results from clinical studies on Fingolimod show that agonism of S1P receptors transiently lowers heart rate and SBP while a chronic treatment may increase BP by 1–3 mmHg. These effects could be attributed to the agonism and functional antagonism of Fingolimod towards S1PR1. To translate our findings into human population we quantified S1P level in a cohort of 82 subjects and found that S1P correlates with a worse endothelial function, as measured by lnRHI parameter. Moreover, an association of S1P level with ASP and AIx suggests that S1P may be a marker of vascular stiffening, although, no correlation was found with a PWV as the main indicator of arterial stiffness. It remains to be explored whether there is a causal relationship between S1P and the above parameters, a marker of vascular stiffening, although, no correlation was found with a PWV as the main indicator of arterial stiffness.

Our study also showed for the first time lower cardiac hypertrophy and changes in aortic superoxide anion production following Ang II infusion in Sphk1\(^{−/−}\) mice. Therefore it opens another possible mechanism of end-organ protection in hypertension. Reduction in cardiac hypertrophy may reflect lower systolic blood pressure but the extent of protection suggests additional mechanisms. Indeed S1P has been reported to be involved in cardiac hypertrophy and defect in models of heart failure. Reactive oxygen species and especially superoxide anion are known to impair endothelial function and contribute to vascular pathology. Several sources of superoxide anion in vasculature have been identified so far and include uncoupled eNOS or NADPH oxidases, among which NOX1 and NOX2 are thought to contribute to vascular pathology the most significantly. Although studies linking S1P pathway with Nox2-mediated vascular redox imbalance in vivo are limited, it has been shown that sphingosine kinases regulate Nox2 activity via intracellular calcium store depletion and further S100A8/A9 translocation in neutrophils. Our study suggests that there is a higher expression of Nox2 NADPH oxidase in both aortic and mesenteric compartments of hypertensive Sphk1\(^{−/−}\) mice as compared to WT mice. This may result in abnormally elevated level of superoxide anion production in Sphk1\(^{−/−}\) mice in hypertension, yet our data only partially confirm this hypothesis. Since we did not find a significantly different level of superoxide anion...
production between hypertensive Sphk1−/− and WT mice using DHE-based method, further work is required to determine effects of Sphk1 deficiency on redox balance in vascular tissues.

While we have focused on Sphk1 as a key driver of several pathways modified in Ang II-induced hypertension, our study has identified several genes and can provide a reference for future in-depth studies. The availability of expression of the whole transcriptome allowed many types of analytical strategies to be employed in order to find genes important for the development of Ang II-induced hypertension. Sphk1 was identified via gene set and a subsequent leading edge analyses, however, this type of analysis identified other genes that possess a known, important a role in BP regulation and vascular biology. T-cadherin (Cdh13), which was present in the high-est number of significantly Ang II-affected gene sets in the thoracic aorta, is known to serve as an adipoectin receptor providing cardioprotective functions. Variations in CDH13 locus were also associated with metabolic syndrome, adiponectin levels and BP in humans. Sphk1−/− mice have impaired vascular remodeling due to low NO bioavailability, and SOD1 is highly expressed and functionally important in human vascular tissue. An attempted follow-up of the loci known to associate with BP in GWAS, identified subunits of soluble guanylate cyclase and Cnm2 among transcripts that expression was the most significantly different between hypertensive and normotensive mice. Soluble guanylate cyclase is a sensor of NO in VSMC, sensitive to Ang II stimulation, and its function in the regulation of BP is well established, while Cnm2 is relatively less studied in the context of hypertension and may be involved in maintaining Mg2+ balance in blood vessels. Of importance variations in the coding region of 5PR1 locus has been recently investigated in a large cohort with respect to their functional role. Interestingly, one rare variant was associated with protection from coronary artery disease, which implicates that variations in the function of SIP-5PR1 pathway may be of clinical significance.

In summary, vascular transcriptome profiling employed in the present study, allowed to identify gene causally related to the development of vascular dysfunction in vivo. S1P pathway stimulation in endothelium protects against vascular dysfunction, however, inhibition of SIP pathway in VSMC decreases vasoconstriction and is likely responsible for the observed lower BP in Sphk1−/− mice following Ang II infusion, independently of endothelial function. Further studies are needed to explore the role of SIP1 in vascular redox balance in Ang II-induced hypertension. Modulation of the Sphk1 activity as well as targeting SIP receptors may provide a promising strategy for future studies aiming to prevent development of severe hypertension.

Methods

Chemicals. Ach, Phe, SNP, hydralazine hydrochloride, lucigenin, noradrenaline, and Ang II used for induction of hypertension in vivo were purchased in Sigma-Aldrich (Germany). Prostaglandin F2α (PGF2α), JTE-013 (S1PR2 antagonist), SIP, and Sphk1 inhibitor PF-543 were purchased in Cayman Chemical (USA).

Experiments in vivo. Male C57BL/6J (n = 24) mice were used to assess the effects of chronic Ang II infusion on vascular transcriptome profile. All mice were bred in SPF animal facility. Sphingosine kinase 1 knockout (Sphk1−/−) (stock No: 019095) and appropriate WT control mice (stock No: 005304; C57BL/6NJ) were obtained from Jackson Laboratory. Twelve-week-old mice underwent either sham or Ang II (490 ng/min/kg/s.c.) treatment for 14 days, via a surgically implanted osmotic minipump (Alzet Model 2002, Alzet Corporation, CA, USA). Sham treatment involved infusion of the vehicle for Ang II. During treatment, all mice underwent non-invasive blood pressure measurement by tail-cuff plethysmography (Visitech BP 2000 BP Analysis System), followed a period of training before commencement of the treatment protocol. Two weeks after pump implantation mice were sacrificed and tissues, i.e. aorta, 2nd and 3rd order branches of superior mesenteric artery and heart were collected. Genotype of Sphk1−/− mice was confirmed using real-time PCR and specific primer/probe set targeting Sphk1. To investigate the dependence of studied Sphk1 responses to initial BP elevation, in a subset of animals hydralazine was co-administered ad libitum in drinking water at an approximate 5 mg/kg/day dose. Mice received hydralazine for two weeks starting 1 day before osmotic minipump implantation. Effects of chronic SIP infusion on vascular function and BP were tested in normotensive mice receiving SIP solution in osmotic minipump for 12 days at a 300 nmol/kg/day dose. All experiments were performed according to relevant guidelines and Local Ethics Committee no. 1 in Kraków (Poland) approved the protocols employed (permissions no. 15/2012, 100/2013, 254/2015 and 157/2016).

Transcriptome profiling. Isolated vessels were carefully cleaned of surrounding tissues and immediately placed in RNA-later solution (Ambion, USA). Two tissues of the same type and treatment status were pooled and RNA was isolated from such pools using Microarray RNEasy kit (Qiagen, USA), treated with DNase I and quantified using Ribogreen reagent (Thermo Fisher Scientific, USA). Amplification and biotinylation of RNA were performed using Illumina Totalprep Amplification kit (Ambion, USA). The quality of RNA and cRNA was tested using Tapestation 2200 instrument (Agilent, USA), which resulted in the removal of samples not fulfilling quality criteria. Hybridization of cRNA to an Illumina WG-6 v2.0 chip was performed according to the manufacturer protocol. Arrays were scanned on HiScan scanner (Illumina, USA). Recent GWAS that identified 29 loci associated with BP1 was used to check for an overlap between these loci and transcripts identified as significantly down- or upregulated in the current study. Transcriptomic data have been uploaded to the GEO server (series no. GSE75815).

Quantification of mRNA expression. Vascular RNA from selected tissues of WT and Sphk1−/− mice was isolated using Microarray RNEasy kit (Qiagen, USA). In an independent experiment thoracic aortas of hypertensive and normotensive WT mice were mechanically separated into 2, adventitia and intima-media, layers using surgical dissecting forceps under microscope without enzymatic treatment. Intima-media layer was then briefly perfused with 300 μl of Qiazol (Qiagen, USA) from the inside and the resulting eluate represented
endothelial-enriched RNA fraction used for RNA isolation. The latter was characterized by, on average, 45x higher expression of Platelet/endothelial cell adhesion molecule 1 (Pecam1), 15x higher expression of Nitric Oxide Synthase 3 (Nos3) and 140x lower expression of Alpha 2 Actin (Acta2) as compared to the remaining intima fraction. Reverse transcription was performed using 400 ng of RNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, USA). Real-time PCR reactions were performed on the 7900HT instrument (Applied Biosystems, USA) using commercially available TaqMan assays for Sphk1, Sphk2, S1pr1, and S1pr3. Expression of S1pr2 was quantified using QuantiFast Probe Assay (Qiagen, USA). Normalized microarray expression data were used to test the stability of expression of 24 commonly used candidate genes using Normfinder ver. 0.953 software (Supplementary Table 2)54. In this way, TATA box binding protein (Tbp) was selected as a constitutive gene for all real-time PCR experiments performed on a chip and high stability of expression across all tissues examined.

Western blotting. Immunoblotting was used to examine expression of Sphk1. Snap-frozen aortas, hearts and mesenteric arteries were homogenized in lysis buffer (in mmol/L: sodium pyrophosphate 50, NaF 50, NaCl 5, EDTA 5, EGTA 5, HEPES 10, Na3VO4 2, PMSF 50, Triton 100 0.5%, and leupeptin, aprotinin and pepstatin, all 1 mg/mL). Proteins extracted from each lysate (30 μg) were separated by electrophoresis on 12% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane. For immunoblotting, nonspecific binding sites were blocked with 5% Bovine Serum Albumin in Tris-buffered saline solution for 1 hour at room temperature. Membranes were then incubated with specific antibodies (anti-Sphk1 (Cell Signalling, no. 3297), anti-α-tubulin (Abcam, no. ab4074), anti-GAPDH (Abcam, no. ab8245) or anti-β-actin (A3441, Sigma-Aldrich) overnight at 4°C (TBS-T, 5% BSA). After incubation with secondary antibodies for 1 hour at room temperature, signals were revealed with fluorescence using IRDye®800CW donkey anty-rabbit secondary antibodies and quantified densitometrically with Odyssey CIX Imaging system and Image Studio Lite (ver. 5.2) software.

Vascular function studies. Vascular reactivity was measured using 750 TOBS instrument (DMT, Denmark) in isolated 2 mm segments of the aorta in organ chambers as previously described4. Contraction of 2 mm aortic segments in response to increasing concentrations of Phe was tested. Endothelium-dependent relaxations to Ach was measured in similar segments of the aorta in organ chambers as reported4. In some experiments mesenteric arteries were pre-incubated for 30 min. in KREBS buffer containing 5 μM of JTE-013 or 100 nM PF-543, while control rings were incubated with vehicle (0.1% DMSO). Experiments concerning mesenteric arteries and their contraction in response to Ang II or noradrenaline and relaxation in response to Ach were performed using 610 M myograph (DMT, Denmark).

Vascular superoxide production. Superoxide production was measured using lucigenin-enhanced chemiluminescence (LgCL). The harvested aortic segments, stored on ice, were cut open to expose endothelial surface and equilibrated for 30 min in aerated Krebs-HEPES buffer at 37°C. LgCL from intact vessels was measured in 2 ml buffer containing lowconcentration lucigenin (5 μM) using Berthold FB12 luminometer. Superoxide production was expressed as relative light units (RLU) per second per mg of dry weight of the vessel. An alternative, DHE-based method was used to quantify superoxide anion production in aorta homogenates of AngII-infused WT and Sphk1−/− mice (n=5 for both groups)55. Fluorescence measurements were performed in 37°C using Synergy H4 instrument. Protein content in homogenates was normalized using Bradford reagent.

Heart and aorta histology. Aortas and hearts were fixed using formalin solution and stored in 70% EtOH. Fixed tissues were stained using Masson’s trichrome (Sigma-Aldrich, Germany) according to the manufacturer protocol. Image] (ver. 1.49)56 was used to quantify intima-media thickness (IMT) in cross-sectional sections of aorta and area of cardiomyocytes in cross-sectional sections of hearts. We also quantified IMT of cardiac arteries of similar size in the left ventricle (diameter of approximately 100 μm; 1–2 arteries per heart) and percent of fibrotic changes in heart sections using ImageJ.

Quantification of S1P level and Sphk activity. Mouse aortas were homogenized for 10 min. in lysis buffer with protease inhibitor cocktail in TissueLyser LT bead mill (Qiagen, USA). Homogenates were centrifuged for 10 min. (6000 g) and resulting supernatant was used to quantify sphingosine kinase activity using ATP depletion assay according to the manufacturer’s instructions (Echelon Biosciences, USA). Amount of total protein in supernatants was quantified with Bradford reagent (Sigma, Germany) and normalized. Mouse blood was collected from right ventricle using heparin as an anticoagulant. Plasma was collected after 10 min. of centrifugation at 2000 g. Level of S1P in mouse plasma, mouse aortic homogenates (prepared as described above) and human serum was quantified using competitive ELISA test according to the manufacturer instructions (Echelon Biosciences, USA).

Patients characteristics. Eighty two patients (Supplementary Table 3) were recruited in the J. Dietl Hospital in Kraków (Poland) and E. Szczeklik Hospital in Tarnów (Poland). Inclusion criteria included age (40–80 years), at least one, documented and conventional, risk factor to develop atherosclerosis and patient’s informed consent to participate in the study. The most import exclusion criteria included: lack of patient’s consent, positive history of major cardiovascular/lung diseases (such as pulmonary hypertension, ischaemic heart disease, stroke, cancer, chronic heart failure, atrial fibrillation, COPD, bronchial asthma), positive inflammatory status (based on medical documentation or CRP level ≥10 mg/l), steroid or immunosuppressive therapy in the last 5 years, pregnancy, breastfeeding, alcoholism, menopause or post-menopausal period with use of hormonal therapy. RHI was assessed using EndoPAT device (Itamar Medical, Caesarea, Israel). RHI was natural log transformed and was analyzed as a continuous variable as well as binary variable according to the recommended cutoff point (i.e. lnRHI ≥ or < 0.51). A1x, PWV and ASP were assessed using SphygmoCor device (Atcor Medical Pty
error of the mean. P values > 1.4 (mesenteric arteries). All remaining statistical analyses were
tested using Mann-Whitney U test or Spearman’s correlation test. Data are presented as means ±
standard errors of the mean. P values < 0.05 were considered significant.

Statistical analyses. Analyses of transcriptome profiles were performed in R (ver. 3.0.2) using lumi, limma
and gplots packages. RNA transcripts expression intensities were normalized using a robust spline normaliza-
tion method. In order to find RNA transcripts globally affected by Ang II in vivo additional analysis comprising
all samples with adjustment for tissue type was performed. Gene set enrichment analysis was performed using
GSEA software (ver. 2.1.0) and Gene Ontology biological process set. Subsequent leading edge analyses were
performed on gene sets that were either significantly (FDR p < 0.05) enriched in hypertension (thoracic aorta) or
showed normalized hypertension enrichment score > 1.4 (mesenteric arteries). All remaining statistical analyses
were performed in IBM SPSS statistics software (ver. 23). For comparison of the effects of Ang II on parameters in
different groups of mice, we employed two-way ANOVA or Student’s t tests. Analysis of organ chamber experim-
ents was performed using repeated measures ANOVA. Associations between S1P level and clinical parameters
were tested using Mann-Whitney U test or Spearman’s correlation test. Data are presented as means ± standard
error of the mean. P values < 0.05 were considered significant.

References
1. Coffman, T. M. Under pressure: The search for the essential mechanisms of hypertension. Nat Med 17, 1402–1409 (2011).
2. Levy, D. et al. Genome-wide association study of blood pressure and hypertension. Nat Genet 41, 677–687 (2009).
3. Ehret, G. B. et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 478, 103–109
(2011).
4. Kato, N. et al. Meta-analysis of genome-wide association studies identifies common variants associated with blood pressure
variation in east asians. Nat Genet 43, 531–538 (2011).
5. Wain, L. V. et al. Genome-wide association study identifies six new loci influencing pulse pressure and mean arterial pressure. Nat
Genet 43, 1005–1011 (2011).
6. Zeggini, E. et al. Meta-analysis of genome-wide association data and large-scale replication identifies additional susceptibility loci
for type 2 diabetes. Nat Genet 40, 638–645 (2008).
7. Cho, M. H. et al. A genome-wide association study of copd identifies a susceptibility locus on chromosome 19q13. Hum Mol Genet
21, 947–957 (2012).
8. Guzik, T. J. et al. Role of the t cell in the genesis of angiotensin ii induced hypertension and vascular dysfunction. J Exp Med 204,
2449–2460 (2007).
9. Guzik, T. J. et al. Vascular superoxide production by nadph oxidase: Association with endothelial dysfunction and clinical risk
factors. Circ Res 86, E85–90 (2000).
10. Makhnova, N. A., Crowley, S. D., Griffiths, R. C. & Coffman, T. M. Gene expression profiles linked to at1 angiotensin receptors in
the kidney. Physiol Genomics 42A, 211–218 (2010).
11. Larkin, J. J., Frank, B. C., Gavras, H., Sultana, R. & Quackenbush, J. Independence and reproducibility across microarray platforms.
Nat Methods 2, 337–344 (2005).
12. Tsushima, K. et al. Ir3 regulates cardiac fibrosis but not hypertrophy in mice during angiotensin ii-induced hypertension. FASEB J
25, 1531–1543 (2011).
13. Rush, C. et al. Whole genome expression analysis within the angiotensin ii–apriloprotein e deficient mouse model of abdominal
aortic aneurysm. BMC Genomics 10, 298 (2009).
14. Owens, A. P. 3rd et al. Angiotensin ii induces a region-specific hyperplasia of the ascending aorta through regulation of inhibitor
of differentiation 3. Circ Res 106, 611–619 (2010).
15. Zhou, Y., Chen, Y., Dhrskm, W. P., Morris, M. & Periasamy, M. At1b receptor predominantly mediates contractions in major mouse
blood vessels. Circ Res 93, 1089–1094 (2003).
16. Poduri, A. et al. Regional variation in aortic at1b receptor mRNA abundance is associated with contracility but unrelated to
atherosclerosis and aortic aneurysms. PLoS One 7, e48462 (2012).
17. Intengan, H. D. & Schiffirn, E. L. Structure and mechanical properties of resistance arteries in hypertension: Role of adhesion
molecules and extracellular matrix determinants. Hypertension 36, 312–318 (2000).
18. Prewitt, R. L., Rice, D. C. & Dobrian, A. D. Adaptation of resistance arteries to increases in pressure. Microcirculation 9, 295–304
(2002).
19. Petnehazy, T. et al. Angiotensin ii type 1 receptors and the intestinal microvascular dysfunction induced by ischemia and reperfusion.
Am J Physiol Gastrointest Liver Physiol 290, G1203–1210 (2006).
20. Wang, D., Chabratlshvili, T., Borrego, L., Aslam, S. & Umans, J. G. Angiotensin ii infusion alters vascular function in mouse resistance
vessels: Roles of o and endothelium. J Vasc Res 43, 109–119 (2006).
21. Peters, S. L. & Alewijnse, A. E. Sphingosine-1-phosphate signaling in the cardiovascular system. Current opinion in pharmacology 7,
186–192 (2007).
22. Moxon, J. et al. Comparison of the serum lipidome in patients with abdominal aortic aneurysm and peripheral artery disease.
Circulation. Cardiovascular genetics 7, 71–79 (2014).
23. Xing, X. Q. et al. Sphingosine kinase 1/sphingosine 1-phosphate signalling pathway as a potential therapeutic target of pulmonary
hypertension. International journal of clinical and experimental medicine 8, 11930–11935 (2015).
24. Goshkova, I. A. et al. Inhibition of sphingosine-1-phosphate lyase rescues sphingosine kinase 1 knock-out phenotype following
murine cardiac arrest. Life sciences 93, 359–366 (2013).
25. Allende, M. L. et al. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by fty720. The journal of biological chemistry
279, 52487–52492 (2004).
26. Wilson, P. C., et al. Inhibition of sphingosine kinase 1 ameliorates angiotensin ii-induced hypertension and inhibits transmembrane
calcium entry via store-operated calcium channel. Molecular endocrinology 29, 896–908 (2015).
27. Furuya, H. et al. Effect of sphingosine kinase 1 inhibition on blood pressure. FASEB J 27, 656–664 (2013).
28. Coussin, F., Scott, R. H. & Nixon, G. F. Sphingosine 1-phosphate induces creb activation in rat cerebral artery via a protein kinase
0-mediated inhibition of voltage-gated k+-channels. Biochemical pharmacology 66, 1861–1870 (2003).
29. Salomone, S. & Waerber, C. Selectivity and specificity of sphingosine-1-phosphate receptor ligands: Caveats and critical thinking in
characterizing receptor-mediated effects. Frontiers in pharmacology 2, 9 (2011).
Siedlinski, M. et al. Vascular transcriptome profiling identifies Sphingosine kinase 1 as a modulator of angiotensin II–induced vascular dysfunction. Sci. Rep. 7, 44131; doi: 10.1038/srep44131 (2017).
