**Sphingosine Kinase 2 Modulates Retinal Neovascularization in the Mouse Model of Oxygen-Induced Retinopathy**

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**PURPOSE.** Neovascularization is a major cause of blindness in various ocular diseases. Bioactive sphingosine 1-phosphate (S1P), synthesized by two sphingosine kinases (Sphk1, Sphk2), emerged as a key player in a multitude of cellular processes, including cell survival, proliferation, inflammation, migration, and angiogenesis. We investigated the role of Sphk2, S1P, and S1P receptors (S1PR) during retinal neovascularization using the oxygen-induced retinopathy mouse model (OIR).

**METHODS.** Sphk2 overexpressing (tgSphk2) and Sphk2 knockout (Sphk2−/−) mice were used in the OIR model, exposed to 75% O2 over 5 days from postnatal day (P)7 to 12 to initiate vessel regression. After returning to room air, these mice developed a marked neovascularization. Retinae recovered from untreated and treated eyes at P7, P12, P14, and P17 were used for lectin-stained retinal whole mounts, mass spectrometry, and quantitative real-time PCR.

**RESULTS.** tgSphk2 mice showed higher retinal S1P concentrations, accelerated retinal angiogenesis, and increased neovascularization. Expression of S1PR, vascular endothelial growth factor (VEGF)α, and angiopoietin 1 and 2 was differentially regulated during the course of OIR in the different genotypes. Sphk2−/− displayed a markedly reduced retinal angiogenesis and neovascularization as well as decreased VEGFα and angiopoietin expression.

**CONCLUSIONS.** Using genetic models of Sphk2 overexpression or deletion we demonstrate a strong impact of Sphk2/S1P on retinal vasculopathy and expression of vascular growth factors like VEGF and angiopoietin in the retina. Consequently, Sphk2, S1P, and S1PR may offer attractive novel therapeutic targets for ischemic retinopathies.

Keywords: lipid signaling, S1P, OIR, sphingosine kinase 2, S1PRs

Neovascularization is an integral process of impairment and loss of vision in ischemic retinopathies, a diverse group of retinal diseases (e.g., diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusions). Retinal hypoxia caused by retinal vessel occlusion or capillary nonperfusion in retinopathies stimulates the production of angiogenic factors, leading to intraocular neovascularization (NV).1–3 The predominant growth factor associated with physiological and pathological angiogenesis is vascular endothelial growth factor α (VEGFα). Several ocular cell types are known to express VEGFα (e.g., Müller cells, pericytes, ganglion cells, and vascular endothelial cells [ECs]), which plays a critical role in retinal neovascularization.4 Besides VEGFα other important angiogenic factors are angiopoietin 1 (Angpt1) and angiopoietin 2 (Angpt2) with their tyrosine kinase receptor Tie2, expressed on ECs. Angpt1 binding to Tie2 stimulates receptor phosphorylation and subsequently activates endothelial nitric oxide synthase (eNOS), protein kinase b (Akt), and extracellular-signal regulated kinase (ERK). Angpt1/Tie2 signaling facilitates vessel stabilization and maturation and maintains the integrity of blood vessels.1–8 Angpt2 acts as a Tie2 antagonist, competing with Angpt1, and reduces receptor phosphorylation, leading to vessel destabilization.9–13 Angpt2 expression increases in ischemic retina and promotes neovascularization in cooperation with VEGFα. In the absence of VEGFα, Angpt2 stimulates regression of neovascularization and pruning of capillary beds.14,15

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid, is an important signaling molecule involved in the regulation of
various cellular processes, such as cell survival, proliferation, differentiation, angiogenesis, migration, and inflammation. In contrast, ceramides and sphingosine, two precursors of S1P, are mediators of proapoptotic processes. Together with S1P, these sphingolipid mediators maintain a balance, termed the "sphingolipid rheostat," which is decisive for cell fate. Sphingosine kinases are central regulators of this equilibrium and catalyze S1P production from sphingosine by phosphorylation. There are two isoforms of sphingosine kinases (Sphk), Sphk1 and Sphk2, which are ubiquitously expressed in all tissues but differ in expression levels and subcellular localization. Currently, Sphk1 is regarded as an enzyme involved in prosurvival and proliferative cascades while the functions and regulations of Sphk2 are not as well characterized and seem to be context dependent. Known Sphk2 functions involve induction of growth arrest and apoptosis, but also promotion of survival and proliferation. There is little experimental evidence concerning the role of Sphk as a possible target of pharmacologic interventions in retinal diseases. However, S1P is a key regulator of angiogenesis and vessel stabilization and a decisive factor in retinal pathologies, contributing to retinal and choroidal neovascularization. S1P mainly exerts its biological effects via five membrane-bound G protein-coupled receptors, S1PR1 to S1PR5. In addition, Sphk2 inhibition was reported to reduce disease severity in diabetic retinopathy.

Here, we investigated the role of S1P and Sphk2 in retinal vascular development and neovascularization using the oxygen-induced retinopathy model (OIR) in mice. We found that Sphk2 is the prominent Sphk isoform in the mouse retina. Moreover, Sphk2 overexpressing mice (tgSphk2) and Sphk2 knockout mice (Sphk2−/) were used to demonstrate that Sphk2 has a major impact on retinal vascularization. Consequently Sphk2 and its product S1P and S1P receptors may prove promising novel targets for ischemic retinopathies.

Materials and Methods

Animal Procedures

The protocols conformed to the German Protection of Animals Act and the guidelines for care and use of laboratory animals by the local committee (Regierungspraesidium Darmstadt) in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with free access to chow and water in a specified pathogen-free facility with a 12-hour day/12-hour night cycle. Sphk2−/− mice with C57BL/6J background were generated and characterized as previously described. Mice overexpressing the human Sphk2 gene were generated by GenOway (Lyon Cedex, France). Briefly, human Sphk2 transgene controlled by the ubiquitous CAG promoter was introduced into the Hprt locus by homologous recombination. A floxed stop cassette was included between promoter and the cDNA allowing Cre-dependent expression of human Sphk2 gene (Supplementary Fig. S1). Homozygous human Sphk2 mice with C57BL/6J background were bred with B6.FVB-Tg(Ela-Cre)C57BL/6J mice (no. 003724; The Jackson Lab, Bar Harbor, ME, USA), carrying a Cre transgene under the control of the adenovirus EIIa promoter. Breeding with Ela-Cre mice resulted in a deletion of the IoxP-flanked stop cassette and subsequent overexpression of human Sphk2. Homozygous tgSphk2 mice, lacking the stop cassette and the Cre transgene, Sphk2−/−, and wild-type controls (C57BL/6J) for all the experiments were bred from our own colonies.

Oxygen-Induced Retinopathy Model and Retina Whole-Mount Preparation

Retinal neovascularization was observed using the OIR model. Accordingly, neonates together with their mother were exposed to 75% oxygen from postnatal day (P)7 to P12. Upon returning to room air (21% O2) at P12 the relative hypoxia induced NV over the period P12 through P17 in these mice, with P17 as the maximum of NV. Normoxia controls were handled in the same environment but without 75% O2 exposure. At P7, P12, P14, and P17, mice were killed, and eyes were fixed for 1 hour at room temperature in 4% formaldehyde. For whole-mount preparation, eyes were placed under a dissecting microscope and cut open along the ora serrata. After removing cornea and iris, RPE and sclera were separated from the retina, following removal of the lens. Isolated retinae were stained with 100 µl 1 mg/ml TRITC-lectin (BSI) from Griffonia simplicifolia (Sigma Aldrich, Taufkirchen, Germany) in 1% Triton X-100, 1 mM CaCl2, 1 mM MgCl2 in 1X PBS overnight, washed with 1X PBS, and transferred to a microscopic slide. After four radial incisions, retinae were coverslipped with mounting medium (DAKO, Hamburg, Germany).

Analysis of Retinal Angiogenesis, Vaso-Obliteration, and Neovascularization

Vascular density analysis is an important feature to evaluate retinal angiogenesis. AngioTool was used for branching point quantification at P7, indicating vascular density. The two main readouts in OIR are vaso-obliteration (VO) and subsequent NV. VO develops during the hyperoxic phase of the model, and NV follows from P14 onward. The percentage and therefore extent of VO were calculated by comparing the central avascular area to the total retinal area using a photo editing program (Photoshop; Adobe Corp., San Jose, CA, USA). Quantification of NV was done at P14 and P17 on retina whole mounts using a computer-aided technique allowing semiautomated quantification of retinal NV based on Imagej software (National Institutes of Health, Bethesda, MD, USA) together with appropriate plugins and macros.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Retinae from unfixed eyes were used for RNA isolation utilizing the Masterpure DNA and RNA Isolation Kit (Biozymb Scientific GmbH, Oldendorf, Germany) and the corresponding protocol. RNA (1 µg) was transcribed into cDNA using a reverse transcription system (Life Technologies Limited, Paisley, UK). Quantitative real-time PCR (TaqMan) was performed using the Applied Biosystems 7500 Fast Real-time PCR System (Darmstadt, Hessen, Germany). Probes, primers, and the reporter dyes 6-FAM and VIC were from Life Technologies (Darmstadt, Germany). Probes used were Mm00445021_m1 (mouse Sphk2), Mm00456503_m1 (Angpt2), Mm00454822_m1 (Angpt2), Mm00457304_m1 (VEGFα), Mm02619656_s1 (S1PR1), AJVI4VC (S1PR2), Mm02620181_s1 (S1PR3), and Mm00486955_s1 (S1PR4). Cycling conditions were as follows: 95°C for 15 minutes (1 cycle), 95°C for 15 seconds, and 60°C for 1 minute (40 cycles). Threshold cycle (Ct) was calculated by the instrument’s software (7500 Fast System SDS Software version 1.4). Analysis of the relative mRNA expression was performed using the 2−ΔΔCt method. The housekeeping gene GAPDH was used for normalization.
Sphingolipid Quantification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Quantification of sphingolipids was performed by LC-MS/MS. For quantitation, the retina tissue samples were first mixed with 150 μL H2O and the internal standards sphingosine-d7, sphingosine 1-phosphate-d7, and sphinganine-d7 (20 μL of a solution of 500 ng/mL). The mixture was homogenized using a swing mill (Retsch, Haan, Germany) with four zirconium oxide grinding balls for each sample (25 Hz for 2.5 minutes). Afterward, the samples were extracted twice with 600 μL methanol:chloroform:HCl (15:83:2, vol/vol/vol). The collected lower organic phases were evaporated at 45°C under a gentle stream of nitrogen and reconstituted in 50 μL methanol. Afterward, amounts of sphingolipids were analyzed by liquid chromatography coupled to tandem mass spectrometry. A Luna C18 column (150 × 2-mm ID, 5-μm particle size, 100-Å pore size; Phenomenex, Aschaffenburg, Germany) was used for chromatographic separation. The HPLC mobile phases consisted of water-formic acid (100:0.1, vol/vol) (A) and acetonitrile-tetrahydrofuran-formic acid (50:50:0.1, vol/vol/vol) (B). For separation, a gradient program was used at a flow rate of 0.3 mL/minute. The initial buffer composition 60% (A)/40% (B) was held for 0.6 minutes and then within 3.9 minutes linearly changed to 0% (A)/100% (B) and held for 6.5 minutes. Subsequently, the composition was linearly changed within 0.5 minutes to 60% (A)/40% (B) and then held for 4.5 minutes. The running time for every sample (injection volume of 10 μL) was 16 minutes. The MS/MS analyses were performed using a triple quadrupole mass spectrometer API4000 (Sciex, Darmstadt, Germany) equipped with an ESI (electrospray ionization) ion source operated in positive. The analysis was done in Multiple Reaction Monitoring (MRM) mode with a dwell time of 50 ms. Data acquisition was done using Analyst Software V 1.6 and quantification was performed with Multi-Quant Software V 3.0 (both Sciex), employing the internal standard method (isotope dilution mass spectrometry). Linearity of the calibration curve was proven for sphingosine from 0.02 to 5 ng/mg tissue and for S1P from 0.1 to 10 ng/mg tissue; the coefficient of correlation was at least 0.99. Variations in accuracy were less than 15% over the whole range of calibration, except for the lower limit of quantification, where a variation in accuracy of 20% was accepted.

Statistical Analysis

Analysis was performed using Graph Pad Prism (San Diego, CA, USA). Statistical tests performed included 1-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test for significance of differences between multiple groups and Student’s t-test for comparison between two groups. Data are presented as mean ± standard deviation (SD). P < 0.05 was considered statistically significant.

RESULTS

Sphk2/S1P Is Associated With Retinal Vascular Development

Sphk isoforms are ubiquitously expressed but show differences in subcellular localizations and tissue-dependent expression levels.22,25 In the retina of wild-type (WT) mice from P12 to P17, both kinases are expressed, but expression levels of Sphk2 were markedly higher (Fig. 1A). Therefore, we focused on the role of Sphk2, making use of Sphk2+/− and tgSphk2 mice (Supplementary Fig. S1). Overexpression of human Sphk2 in the retina was confirmed by measuring retinal human Sphk2 mRNA expression (Fig. 1B). This mouse mutant is characterized by higher retinal S1P levels.

Branching point analysis of TRITC-lectin-stained retinas from WT, Sphk2−/−, and tgSphk2 mice on P7 showed a prematurely dense vessel network in tgSphk2 in comparison to WT mice, whereas the vessel density was reduced in Sphk2−/− mice (Figs. 2A, 2B).

Sphk2 Overexpression Accelerates Neovascularization in the Hypoxic Phase While Absence of Sphk2 Leads to Defective Vessel Growth

When subjected to OIR, the central area of VO after 5 days of hyperoxia (P12) was significantly reduced in tgSphk2 by 14.9% in comparison to WT mice, whereas Sphk2−/− mice showed a similar avascular area (Fig. 3A, P12). In tgSphk2, coverage of the avascular area by newly formed vessels occurred faster than in WT mice, contrary to Sphk2−/−. At P17, revascularization of the avascular area had progressed further in all three genotypes. In WT mice, the avascular area was reduced by 24.1% from P12 to P17. The initially faster revascularization in tgSphk2 slowed down, with a total reduction of 20.0%,
resulting in a significantly smaller avascular area at P17. In Sphk2<sup>−/−</sup> the avascular area decreased by only 17.2% from P12 to P17 (Fig. 2B). To further investigate these differences in the reduction of the avascular area, the extent of neovascularization was evaluated by relating the area covered by NV to the total retinal area (Fig. 3C) and by observing clusters and tufts of blood vessels. While tgSphk2 showed significantly increased NV in comparison to WT mice at all time points, NV was decreased in Sphk2<sup>−/−</sup>. In both strains, the visual appearance of the blood vessel system clearly differed from WT mice at all time points. In tgSphk2, clusters and tufts were already formed at P14, and at the later stage the blood vessel network had a more chaotic appearance than in WT mice. In Sphk2<sup>−/−</sup> the blood vessel system appeared even more severely affected than in the tgSphk2 retina, and a large number of cluster and tufts were visible (Fig. 3A).

**Retinal S1P Levels Are Not Regulated During OIR**

Retinal S1P levels did not show significant alterations during OIR in WT mice. Under normoxia, retinal S1P concentrations were increased in comparison to WT mice both in tgSphk2 and, counterintuitively, in Sphk2<sup>−/−</sup> mice as has already been described (and attributed to a compensatory upregulation of Sphk1) previously for this mutant.42 OIR did not induce further significant changes of S1P levels (Fig. 4A). Sphingosine, the precursor of S1P, increased significantly in Sphk2<sup>−/−</sup> but not in tgSphk2 mice (Fig. 4B).

**S1P Receptor Expression Is Differentially Regulated During the Course of OIR in the Different Genotypes**

In view of the increased retinal S1P levels in tgSphk2 and Sphk2<sup>−/−</sup> mice, we performed mRNA expression studies on S1PRs. Retinal S1PR expression was detected for four of the five receptors (S1PR1 > S1PR3 > S1PR2 > S1PR4, data not shown). We found a downregulation of S1PR1 at P12 and P14 in OIR in comparison to WT mice of the same age (Fig. 5A). S1PR2 was upregulated at all time points following OIR; S1PR3 did not show significant changes, and S1PR4 was strongly upregulated (20-fold) at P12 and P14 and significantly downregulated at P17 (Figs. 5B–D). S1PR1 showed a lower baseline expression in tgSphk2 and Sphk2<sup>−/−</sup> mice and OIR did not induce significant changes (Fig. 5A). The expression of S1PR2 was also significantly lower in tgSphk2 and Sphk2<sup>−/−</sup>, treatment independent (Fig. 5B). S1PR3 expression was not regulated differentially in tgSphk2 or Sphk2<sup>−/−</sup> (Fig. 5C). Strikingly, S1PR4 expression was 10-fold higher in tgSphk2 as compared to WT mice but did not change significantly upon OIR. At P17, when NV reaches its maximum, S1PR4 levels decreased both in WT and tgSphk2 mice subjected to the
model. The expression of S1PR4 in Sphk2−/− remained very low (Fig. 5D).

**Sphk2 Overexpression Regulates Growth Factor Expression in a Time-Dependent Manner Whereas Sphk2−/− Mice Show a Reduced Capacity to Express Growth Factors**

To elucidate the mechanisms underlying the differential course of VO and NV in tgSphk2 and Sphk2−/−, we performed mRNA expression studies. VEGFα, the initiating factor of neovascularization, was upregulated in OIR from P14 onward in all genotypes compared to their respective normoxia controls. Surprisingly, retinae of tgSphk2 mice that underwent accelerated neovascularization in OIR showed lower VEGFα expression levels than WT mice throughout the whole time course of the model (Fig. 6A). Compared to WT mice, in Sphk2−/− the VEGFα levels were drastically reduced at all time points under normoxia as well as in the OIR model, but still showed a slight increase from P14 onward in OIR (Fig. 6B). Since the acceleration of neovascularization in tgSphk2 could not be explained by increasing VEGFα expression upon hypoxia, we analyzed Angpt1 and Angpt2. In OIR, tgSphk2 mice showed significantly upregulated Angpt1 and Angpt2 levels in comparison to WT mice at P12, which decreased during the hypoxic phase (Figs. 7A, 7C). Angpt1 decreased from P14 until it was down to similarly low levels as observed in WT (Fig. 7A). Angpt2 expression was downregulated from P12 to P14 but still higher than in WT. Interestingly, at P17, Angpt2 levels in WT stayed upregulated, while they were significantly reduced in tgSphk2 (Fig. 7C). In contrast, Angpt1 in Sphk2−/− expression remained low and Angpt2 showed only a moderate upregulation at P17 (Figs. 7B, 7C).

**FIGURE 3.** Effect of genetic modulations of Sphk2 on the degree of vasculopathy in the OIR model. (A) WT, tgSphk2, and Sphk2−/− retina whole mounts from postnatal days 12, 14, and 17 were stained with TRITC (tetramethylrhodamine)-conjugated lectin. Scale bar: 500 μm. (B) The central avascular area is given in % of the total retina area (avascular area bordered by dotted line). (C) NV is given in % of the total retinal area. Results are means ± SD; n = 12/group; *P < 0.05, **P < 0.01, ***P < 0.001.

**FIGURE 4.** Retinal concentrations of sphingosine 1-phosphate (S1P) and sphingosine in WT, tgSphk2, and Sphk2−/− mice in the OIR model. Retina levels of S1P (A) and sphingosine (B) were measured by LC-MS/MS. Results are means ± SD; n = 8/group; *P < 0.05, **P < 0.01, ***P < 0.001.
DISCUSSION

The present study investigates the role of Sphk2 in retinal vascular development and neovascularization. S1P, the catalytic product of Sphk2, is known to participate in stimulating angiogenesis and vessel maturation.43 The functional role of S1P in the retina has been investigated in recent studies, creating evidence for its important contribution to retinal pathologies,29 retinal and choroidal neovascularization,30 and profibrotic and proinflammatory responses in pigment epithelium cells.44,45

We demonstrate that genetic modulation of Sphk2 with corresponding changes of retinal S1P levels leads to a profound alteration of angiogenesis and accelerated neovascularization during OIR. In tgSphk2 we observed an accelerated retinal angiogenesis under physiological conditions and an accelerated but chaotic neovascularization in OIR when compared to WT. In contrast, angiogenesis was slowed down in Sphk2/C0 and neovascularization was severely altered in OIR.

Two recent publications have established S1P signaling via S1PR1 as a critical but not exclusive signal for inhibition of angiogenesis and the achievement of vascular stability during vessel maturation that acts in parallel with Notch signaling but also independent.27,28 S1PR1-deficient mice displayed a hyper-sprouting phenotype confirmed and found to be EC specific,
with S1PR1 expression gradient being low expression in tip cells of the retinal vasculature.\textsuperscript{27} In this context, it was shown that signaling via S1PR1 inhibits VEGF signaling.

The phenotype of our \textit{tgSphk2} mice, with lower S1PR1 expression during physiological vessel development until P14, appears similar to that of S1PR1-deficient mice and showed an accelerated angiogenesis with neovascular tufts resembling the angiogenic hypersprouting seen in these mice. In contrast, \textit{Sphk2}\textsuperscript{-/-} mice showed opposite effects concerning retinal vascularization, and slightly higher S1PR1 expression at P12 under normoxia compared to \textit{tgSphk2}. Therefore, differential expression of the S1PR1 could be a critical determinant of the neovascularization phenotype of the two mutant strains. Among the two isoforms of Sphk, we found Sphk2 to be more prominent in the retina. This has been described in other neuronal tissues as well.\textsuperscript{46}

We found that the retinal vasculature of \textit{tgSphk2} mice is less sensitive to vessel loss in hyperoxia, which may be due to an accelerated vessel maturity in comparison to WT at the time point investigated. However, the neovascularization that occurs in the hypoxia phase is more chaotic. By contrast, \textit{Sphk2}\textsuperscript{-/-} mice show a delayed retinal angiogenesis and have a catastrophic outcome in the OIR model with very sparse and chaotic neovascularization.

Extracellular S1P regulates a wide range of cellular processes and physiological functions via its GPCRs, including the vascular system.\textsuperscript{32} During the hyperoxia phase of OIR, a significant downregulation of S1PR1 and an upregulation of S1PR2 occur. Generally, S1PR1 protects vascular integrity in many pathologic states, whereas S1PR2 is presumed to mediate vascular damage and endothelial barrier dysfunction.\textsuperscript{33} Concerning the role of S1PR1 as an angiogenic stop signal that has been recently discovered, a rapid downregulation of S1PR1 starting at P12, when the mice are transferred from hyperoxia to room air and neovascularization sets in to compensate for the vessel loss, may be one mechanism to unleash vessel regrowth in the avascular area. The most strikingly regulated S1P receptor, however, was S1PR4. This is a novel and somewhat unexpected finding as S1PR4 has been hitherto known as an S1PR on lymphoid cells involved in the regulation of immune responses. However, our group has found a significant expression on brain endothelial cells (data not shown) and we propose that S1PR4 acts in concert with S1PR1 and S1PR2 and, in the case of \textit{tgSphk2} mice, might be massively upregulated to compensate for the downregulation of S1PR1. Overall, the changes we observed in S1PR1 and -2 in relation to S1PR4 expression suggest a potential compensatory response by S1PR4. But also, S1PR1-independent functions of S1PR4 in angiogenesis should be further investigated. Our data

\textbf{FIGURE 7.} Angiopoietin 1 and 2 expression in \textit{tgSphk2} and \textit{Sphk2}\textsuperscript{-/-} mice. Angiopoietin 1 (Angpt1) and angiopoietin 2 (Angpt2) mRNA expression was measured by qRT-PCR in retinas of WT, \textit{tgSphk2}, and \textit{Sphk2}\textsuperscript{-/-} mice under normoxia and in the OIR model. (A) Angpt1 levels in \textit{tgSphk2} and WT mice. (B) Angpt1 expression in \textit{Sphk2}\textsuperscript{-/-} and WT mice. (C) Angpt2 levels in \textit{tgSphk2} and WT mice. (D) Angpt2 expression in \textit{Sphk2}\textsuperscript{-/-} and WT mice. Results are means ± SD; \(n = 6\)/group; *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).
Sphingosine Kinase 2 Modulates Neovascularization

Indicate a possible functional role and an interplay of S1P receptors in OIR in the retina that is modulated by overexpression of Sphk2. To the best of our knowledge, there are no reports on a direct regulation of S1PR expression by either Sphk1 or Sphk2.

Since the level of oxygen is the main driver of the pathophysiologic processes in OIR, we investigated the hypoxia-driven signaling molecules VEGFα and Angpt1 and -2 in the context of Sphk2 modulation. VEGFα acting via VEGF receptor 2 (VEGFR2) lays a critical role in retinal angiogenesis and neovascularization. It acts in concert with the Tie2 pathway. Tie2/Angpt1 activation leads to vessel stabilization and maturation, whereas Angpt2 contributes to developmental vessel sprouting and vessel destabilization, and both are needed for new blood vessel formation. In contrast, Angpt1 suppresses retinal and choroidal neovascularization.

Interestingly, our tgSphk2 mice despite their phenotype did not show significant differences in VEGFα levels. However, at P12 following the hypoxia phase, they showed higher levels of Angpt2 and also a higher expression of the stabilizing Angpt1, working hand in hand with VEGFα to mediate angiogenesis, leading to enhanced angiogenesis, maturation, and reduced vessel loss. Under hypoxia, tgSphk2 mice showed a similar drop in Angpt1 as WT mice, but an accelerated time course of Angpt2 expression with an early upregulation in prenatal stages of VEGFα to induce neovascularization and a faster return to baseline levels, which may explain the observed excess of neovascularization.

Sphk2−/− mice showed a dramatic deficiency in VEGFα expression, and Angpt1 and Angpt2 expression both under normoxia and under OIR. The downregulation of Angpt1 and Angpt2 leads to a delayed retinal vascularization. Hypoxia hardly triggered an increase of VEGFα, Angpt1, and Angpt2 expression in this genotype. Only the expression of Angpt2 increased at the later time point of the hypoxia phase, but this is inefficient since in the context of low VEGFα levels, Angpt2 has been shown to promote regression of neovascularization. This may explain the severely defective vessel regrowth in Sphk2−/−.

Interaction between S1P signaling pathway and vascular growth factors has already been described. S1P has been shown to transactivate the VEGFR, even in the absence of VEGFα, indicating S1P and VEGFα acting in concert concerning certain signaling functions, and may explain some of the (sparse) retinal neovascularization seen in OIR in Sphk2−/−. On the other hand, S1PR1 signaling has been shown to mediate intracellular effects of VEGFα transduced by VEGFR2, a mechanism assumed to underlie the limitation of angiogenic sprouting by S1PR1. The apparent discrepancy of the reported findings may result from the pleiotropic actions of S1P and the potentially opposing downstream effects of the different S1PRs. Even though the modulation of retinal S1P concentrations as measured by LC/MS/MS appears to be only moderate, local paracrine receptor-mediated and intracellular signaling processes that are not reflected in tissue S1P level could be active. We found a strong interplay between Sphk2 and S1PR expression associated with an impressive alteration of the disease course in OIR. Our data suggest that increase Sphk2 activity and loss of S1PR1 lead to accelerated neovascularization. Concerning S1P-directed therapy, so far mainly an S1P neutralizing antibody (sonepcizumab) has been tried in animal studies, and first clinical trials in humans yielded contradictory results (positive in mice, neutral in humans).

Therefore, stage-specific pharmacologic inhibition of Sphk2 and S1P receptors may offer therapeutic potential to treat retinal diseases involving neovascularization, for example, diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion.

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References

15. Yang L, Yue S, Liu X, Han Z, Zhang Y, Li L. Sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis is involved
Sphingosine Kinase 2 Modulates Neovascularization


