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Research Article

Mechanism of sphingosine 1-phosphate clearance from blood

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The interplay of sphingosine 1-phosphate (S1P) synthetic and degradative enzymes as well as S1P exporters creates concentration gradients that are a fundamental to S1P biology. Extracellular S1P levels, such as in blood and lymph, are high relative to cellular S1P. The blood-tissue S1P gradient maintains endothelial integrity while local S1P gradients influence immune cell positioning. Indeed, the importance of S1P gradients was recognized initially when the mechanism of action of an S1P receptor agonist used as a medicine for multiple sclerosis was revealed to be inhibition of T-lymphocytes' recognition of the high S1P in efferent lymph. Furthermore, the increase in erythrocyte S1P in response to hypoxia influences oxygen delivery during high altitude acclimatization. However, understanding of how S1P gradients are maintained is incomplete. For example, S1P is synthesized but is only slowly metabolized by blood yet circulating S1P turns over quickly by an unknown mechanism. Prompted by the counterintuitive observation that blood S1P increases markedly in response to inhibition S1P synthesis (by sphingosine kinase 2 (SphK2)), we studied mice wherein several tissues were made deficient in either SphK2 or S1P degrading enzymes. Our data reveal a mechanism whereby S1P is de-phosphorylated at the hepatocyte surface and the resulting sphingosine is sequestered by SphK phosphorylation and in turn degraded by intracellular S1P lyase. Thus, we identify the liver as the primary site of blood S1P clearance and provide an explanation for the role of SphK2 in this process. Our discovery suggests a general mechanism whereby S1P gradients are shaped.

Introduction

Sphingosine 1-phosphate (S1P) is a signaling molecule that is abundant in circulation relative to tissue parenchyma. The differential between circulating and tissue S1P is required both for correct lymphocyte positioning [1,2] and to support endothelial barrier integrity [3–5]. Indeed, Xiong and Hla have suggested that the blood — tissue S1P gradient may be intrinsic to all closed circulatory systems [6]. The clinical success of S1P receptor modulators as immune-suppressant medicines for multiple sclerosis is a function of these drugs preventing lymphocyte recognition of S1P gradients [7]. However, knowledge of the mechanisms whereby these gradients are shaped is incomplete.

S1P concentrations in plasma range from 0.1 to 0.8 μM [8] while whole blood levels are considerably higher [9], primarily due to the high S1P levels in erythrocytes [10,11]. Tissue S1P levels are considerably lower than those in blood [12], although tissue S1P measurements are made problematic by blood contamination, rapid metabolism and matrix ionization suppression (S1P is usually measured by LCMS). Like all tissues, blood synthesizes S1P, but it is unusual in its paucity of S1P degrading enzymes [13], which accounts for the high accumulation of S1P in blood. The marked increase in erythrocyte S1P in human volunteers taken to extremely high altitude (5200 m (10% oxygen)) was recapitulated in laboratory mice housed in an 10% oxygen environment [14]. In these mice, the

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increased erythrocyte S1P was found to drive the increased 2,3-bisphosphoglycerate accumulation that is necessary for high altitude acclimatization. We observed subsequently that increasing erythrocyte S1P levels in normoxic mice by administration of an SphK2 inhibitor resulted in similar hemodynamic changes [15].

Perhaps less appreciated than the existence of a blood-S1P gradient is the rapidity whereby circulating S1P turns over. Traceable S1P (such as C_{17}-S1P) injected intravenously (i.v.) into mice is cleared with a $t_{1/2}$ of ~15 min [16–19]. Such observations are consistent with the rapid (min) changes in circulating S1P that are observed following i.v. administration of either SphK1 [20] or SphK2 inhibitors [19]. Because S1P levels in blood *ex vivo* are stable for hours [16], another tissue such as liver and/or endothelium is somehow removing S1P from blood.

Diminished SphK1 activity, whether accomplished by genetic manipulation [9,21] or administration of an isoform selective inhibitor [20], results in decreased blood S1P. This response is congruent with genetic studies indicating that erythrocytes are the primary source of plasma S1P [2] and have only SphK1. What is not readily explained are the observations that *Sphk2*$^{-/-}$ mice exhibit blood, serum and plasma S1P levels that are 2–3 times higher than control mice [9,22–24] and that this phenotype is recapitulated by the administration of SphK2 inhibitors to either mice or rats [9,19].

Prompted by the paradoxical observation that deficiency of SphK2 results in increased levels of its product in blood as well as by a general lack of understanding of mechanisms of S1P clearance from blood, we undertook the study reported herein. Using a set of genetically modified mice, we identified the liver as the predominant site for clearance of S1P from blood and plasma. Our results are consistent with a model whereby S1P is de-phosphorylated at the hepatocyte surface to yield sphingosine that is in turn captured via an SphK2-catalyzed phosphorylation event regenerating S1P that is finally degraded by S1P lyase.

Materials and methods

Mice

The mice used in this study were the C57BL/6 strain. Mice of both genders, in approximately equal numbers, were used throughout the study. The mice used were 6–10 weeks of age. Mice with a floxed sphingosine kinase type 2 (*Sphk2*) allele were made under contract by Ingenious Targeting Laboratories (Ronkonkoma, NY) on a C57BL/6 background. Wild-type C57BL/6J mice were purchased initially from Jackson Laboratories (Bar Harbor, ME) and were used to establish our C57BL/6 colony. Muscle creatine kinase (MCK) Cre, SRY-box containing gene 2 (Sox2) Cre and Albumin (Alb) Cre mice were from Jackson laboratories. All animal experimentation was performed at the University of Virginia and was pre-approved by the University of Virginia's Animal Care and Use Committee. Prior to drawing blood from the retro-orbital sinus, mice were anesthetized to effect using isoflurane.

Tamoxifen induction

To force expression of Cre recombinase in the endothelium, we injected (intraperitoneal route) *Sphk2*$^{fl/fl}$ *Cdh5-ER*^{T2}-Cre mice [25] with tamoxifen (10 mg/ml, 0.1 ml) or vehicle (peanut oil) once daily for 10 days. Mice were bled 10 days after the final injection.

Recombinant adenovirus

Human SphK1, SphK2, and catalytically inactive SphK2(D211A) (numbering refers to isoform c, NCBI accession # NP_001191089) cDNAs containing a carboxyl-terminal V5 epitope tag were sub-cloned into the pAdTRACK-CMV shuttle vector and adenoviruses (subclass C, serotype 5) were generated using the pAdEASY system as described previously [26,27]. A lack of kinase activity of the mutant SphK2(D211A) construct was verified using our yeast-based sphingolipid kinase assay [28]. The Cre recombinase expressing adenovirus was originally from Baylor College of Medicine Gene Vector Core laboratory. High-titer virus for tail vein injection was purified by banding in a CsCl gradient followed by dialysis against phosphate buffered saline (PBS). Hepatic expression of sphingosine kinases or Cre recombinase was accomplished by injecting 0.1 ml of purified recombinant adenovirus in phosphate buffered saline into the tail vein. Blood was drawn 3 days post injection for sphingosine kinase-expressing adenoviruses and 7–10 days post injection for Cre recombinase encoding adenovirus. The extent of adenovirus infection at 3 days post injection was determined in several animals by examining liver slices for green fluorescent protein expression.

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Blood counts

Complete blood cell counts were obtained from 15 μl of mouse blood using a Heska HT5 Element blood analyzer.

Western blot

Liver was collected and homogenized with a motor driven homogenizer in PBS buffer with proteinase inhibitors. Following centrifugation (10 min at 1000×*g*), the supernatant fluid from 1 mg of liver was displayed by polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with antibody. The anti-SphK2 antibody was purchased from Proteintech (catalog # 17096-1-AP, lot # 00048582) and used at a dilution of 1 : 2000. The affinity purified anti-Plpp3 rabbit antisera was a gift from Dr. Andrew Morris (Univ. Kentucky) and was used at a 1 : 3000 dilution. The secondary antibody was an anti-rabbit IgG, HRP-linked antibody (Cell Signaling catalog #7074s).

Liquid Chromatography–Mass Spectrometry (LCMS)

Blood samples were drawn from the retro-orbital sinus and aliquots (blood 10 μl, plasma 10 μl) were prepared for LCMS using the sample preparation protocol of Shaner et al. [29]. Before processing, 10 pmole of deuterated S1P was added as an internal standard (S1Pd7, (Avanti Polar Lipids, Alabaster, AL)). Immediately prior to LCMS analysis, the dried material was dissolved in 0.3 ml of methanol, clarified by centrifugation and a 50 μl aliquot of the supernatant fluid was injected on column. Analyses were performed using a triple quadrupole mass spectrometer (AB-Sciex 4000 Q-Trap) with a Shimadzu LC-20AD inlet. A binary solvent gradient with a flow rate of 1 ml/min on a reverse phase Supelco Discovery C18 column (50 mm × 2.1 mm, 5 μm bead size) was used. Mobile phase A consisted of water : methanol : formic acid (79 : 20 : 1) while mobile phase B was methanol : formic acid (99 : 1). The run started with 100% A for 0.5 min. Solvent B was then increased linearly to 100% B in 5.1 min and held at 100% for 4.3 min. The column was finally re-equilibrated to 100% A for 1 min. S1P was detected using multiple reaction monitoring (MRM) protocols as follows: S1P (380.4 → 264.4), deuterated S1Pd7 (387.4 → 271.3), in positive mode using the following voltages, DP: 76; EP: 10; CE: 29; CXP: 10. Quantification was carried out by comparing S1P and S1Pd7 peak areas using the AB Sciex software Analyst ver. 1.7. Additional analyses were performed using a tandem quadrupole mass spectrometer (Waters Xevo TQ-S micro) with a Waters Acquity UPLC (h-class+) inlet. The liquid chromatography protocol was adapted from Frej et al. [30]. A binary solvent gradient with a flow rate of 0.4 ml/min on a reverse phase C18 UPLC column (Waters CSH C-18 1.7 μm bead size, 2.1 mm × 100 mm) was used. A volume of 3 μl was injected on column. Mobile phase A consisted of water : methanol : formic acid (79 : 20 : 1) while mobile phase B was methanol : acetone : water : formic acid (68 : 29 : 2 : 1). The run began with 50 : 50 A : B for 0.5 min. Solvent B was then increased linearly to 100% B in 3.5 min and held at 100% B for 3 min. The column was re-equilibrated to 50 : 50 A : B for 1.5 min. Analytes were detected using MRM protocols as follows: S1P (380.1 → 264.4, voltages: cone 18, collision 16), deuterated S1Pd7 (387.2 → 271.4, voltages: cone 24, collision 16), sphingosine (300.3 → 252.3, voltages: cone 30, collision 18) in positive mode. Quantification was accomplished using Waters MassLynx ver. 4.1 software. In all cases, the LCMS operator was blinded to the identity of the samples analyzed.

Results

S1P circulates either bound to plasma proteins or inside cells (most prominently in erythrocytes) and while circulating S1P turns over rapidly, S1P is only very slowly metabolized in blood *ex vivo* (*ibid.*). Therefore, we hypothesized that the tissues in direct contact with blood, e.g. endothelium and liver, are the most likely sites responsible for the clearance of blood S1P. Our initial focus was on SphK2 due to the intriguing observation that blockade of this S1P synthetic enzyme results in elevated blood S1P. To enable tissue specific deletion of *Sphk2,* we used a C57BL/6 mouse strain with floxed *Sphk2* alleles (Figure 1A). Germ line cre recombinase catalyzed deletion of three *Sphk2* exons bounded by *loxP* sites to generate SphK2 null ($Sphk2^{-/-}$) mice. Consistent with previous reports [8,22–24] of other $Sphk2^{-/-}$ mice strains, these SphK2 null mice had significantly higher circulating S1P levels than $Sphk2^{fl/fl}$ mice (Figure 1B).

Mice deficient in SphK2 in liver (hepatocytes) were generated by expressing Cre recombinase under the control of the albumin promoter (Alb-Cre) in $Sphk2^{fl/fl}$ mice or by injecting $Sphk2^{fl/fl}$ mice with hepatotropic adenovirus [31] encoding cre recombinase. SphK2 deficiency in hepatocytes resulted in significantly higher

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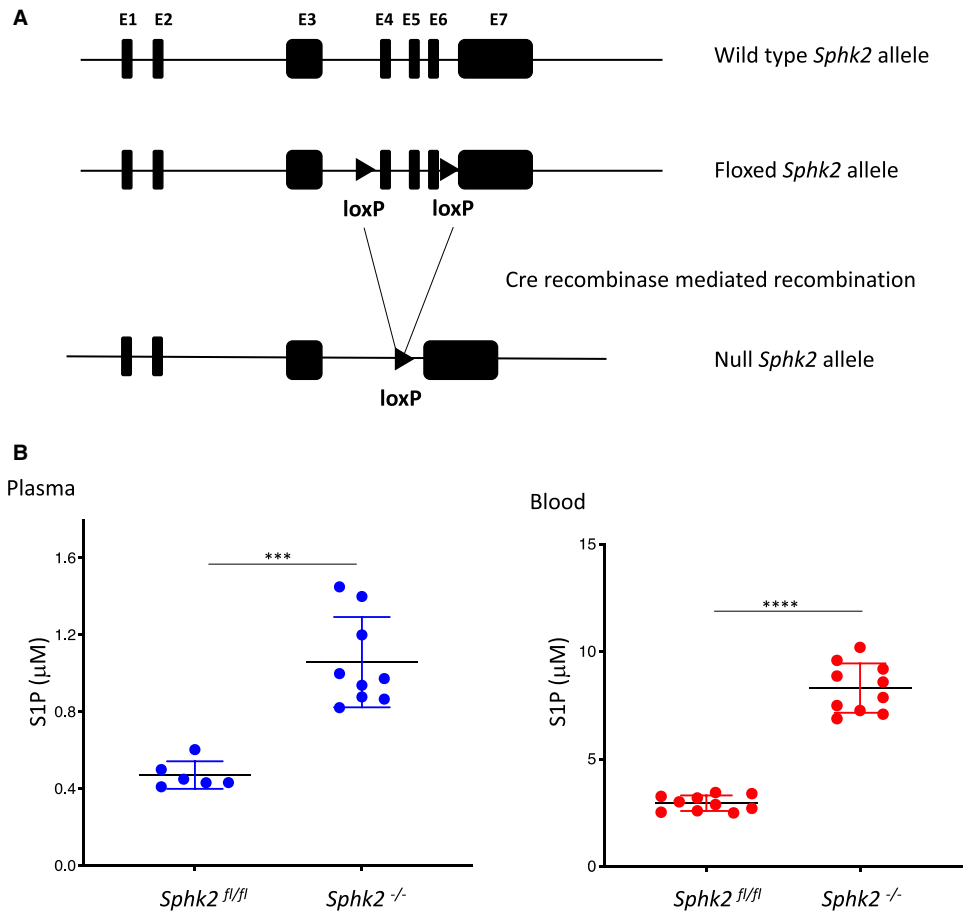

Figure 1. Schematic diagram of the mouse SphK2 gene.

(**A**) The wild-type SphK2 allele (top) showing exons 4–6 flanked by *loxP* sites (middle) and the resulting SphK2-null allele (bottom) (realized after crossing with a *Sox2* Cre deleting mouse). (**B**) Plasma (blue circle, left) and whole blood (red circles, right) S1P levels from SphK2 mice with floxed or null *Sphk2* alleles (congenic on a C57BL/6 background, $n = 6–10$, 6–8 weeks of age, both sexes represented). S1P quantification methodology is described in the *Materials and Methods* section. Student's *t*-test was used for estimating statistical significance between paired groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

blood and plasma S1P as compared with control mice (Figure 2A). The elimination of detectable SphK2 in liver extracts of *Sphk2*$^{-/-}$ mice as well as the efficiency of *Sphk2*$^{fl/fl}$ deletion in liver by Cre recombinase are documented in Figure 2B. Mice deficient in SphK2 in endothelium were generated by tamoxifen injection into *Cdh5-ERT2-cre Sphk2*$^{fl/fl}$ mice. SphK2 deficiency in endothelium resulted in significantly higher circulating S1P (Figure 2C), but the effect was less pronounced than in mice with SphK2 deficiency in liver. However, even in liver SphK2-deleted mice, the rise in circulating S1P was not as large as in global SphK2 deleted (null) mice (compare Figure 1B with Figure 2A). This could be due to the contribution of other tissues as well as the incomplete gene deletion that is inherent to using cre recombinase.

Our observation that SphK2 deficiency in hepatocytes alone results in substantially increased circulating S1P prompted us to ask whether the opposite was true. That is, would forcing expression of SphK2 in hepatocytes of *Sphk2*$^{-/-}$ null mice, which have elevated circulating S1P, be sufficient to normalize blood S1P levels? To do this experiment, we introduced SphK2 in liver by again taking advantage of hepatotropic nature of adenovirus. Recombinant adenovirus strains encoding either wild type or catalytically inactive (D211A) human SphK2 were introduced via tail vein injections into *Sphk2*$^{-/-}$ mice. As illustrated in Figure 3A, administration of a virus encoding SphK2 significantly decreased plasma and blood S1P while the enzymatically inactive SphK2 was without effect. To learn whether this effect was peculiar to the SphK2 isoform, we injected recombinant

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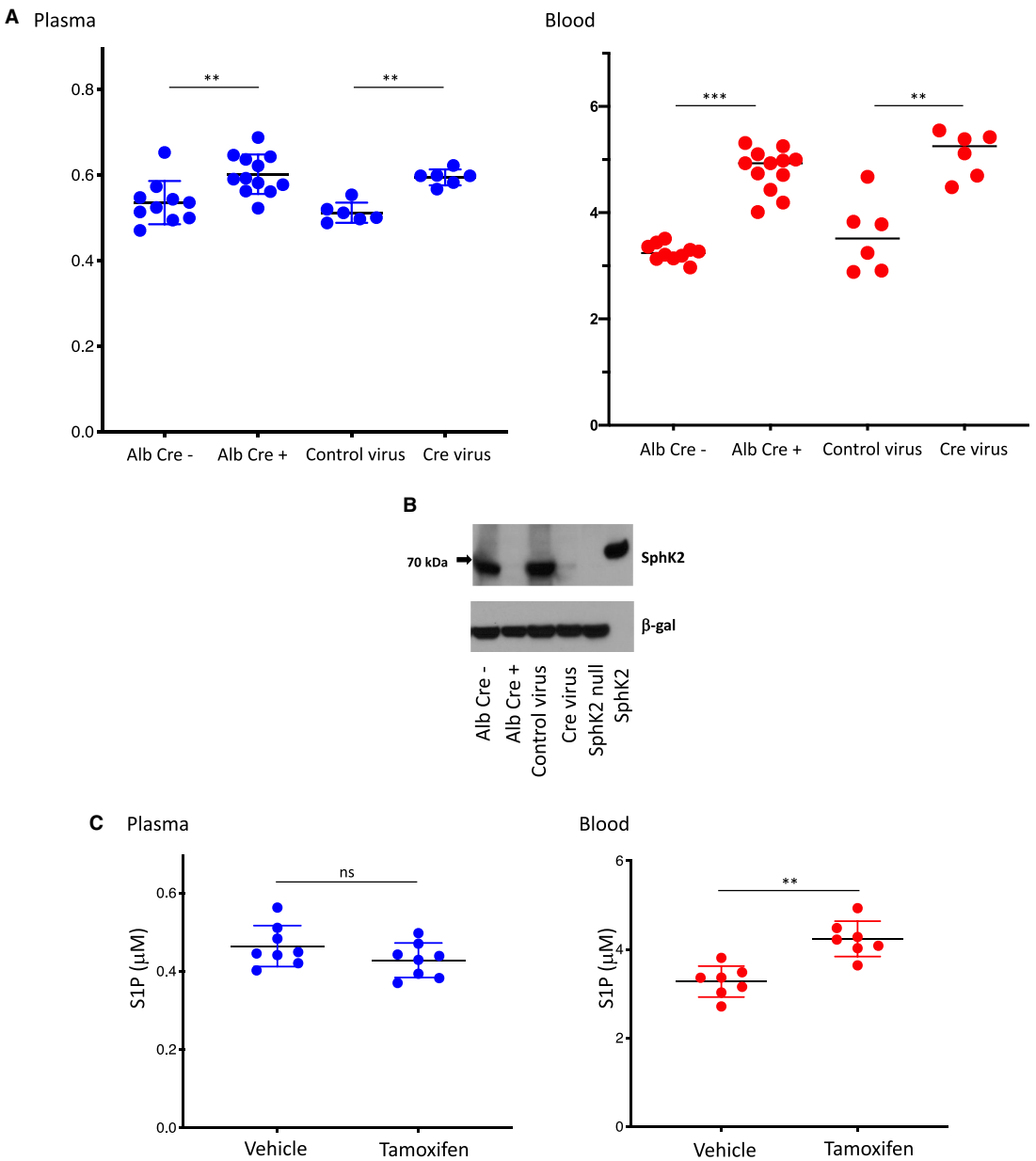

Figure 2. Plasma (blue circles) and blood (red circles) S1P levels in mice with tissue-specific deletion of *Sphk2*.

(**A**) *Sphk2*$^{fl/fl}$ mice were bred with (**A**) *Alb-cre* mice or injected with recombinant (Cre) adenovirus to generate hepatocyte specific deletion, (**B**) Western blot analysis of mouse liver as indicated. The material in the right hand lane is recombinant mouse SphK2. (**C**) *Sphk2*$^{fl/fl}$ mice were bred with *Cdh5-ERT2*-cre mice to generate endothelium specific deletion after tamoxifen treatment. Student's *t*-test was used for estimating statistical significance between paired groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

adenovirus encoding human SphK1 into *Sphk2*$^{-/-}$ mice, and found that circulating S1P was also reduced in these mice (Figure 3B). Notably, forced expression of neither SphK1 nor SphK2 in the livers of wild-type C57BL/6 mice affected whole blood or plasma S1P levels (Figure 3C). In sum, these results emphasize the commonality of SphK1 and SphK2 and buttress the notion that it is the enzymatic activity of SphK2 that is required to normalize blood S1P levels. The predominance of SphK2 in clearing circulating S1P is a reflection of that isoform's greater expression in the liver [32].

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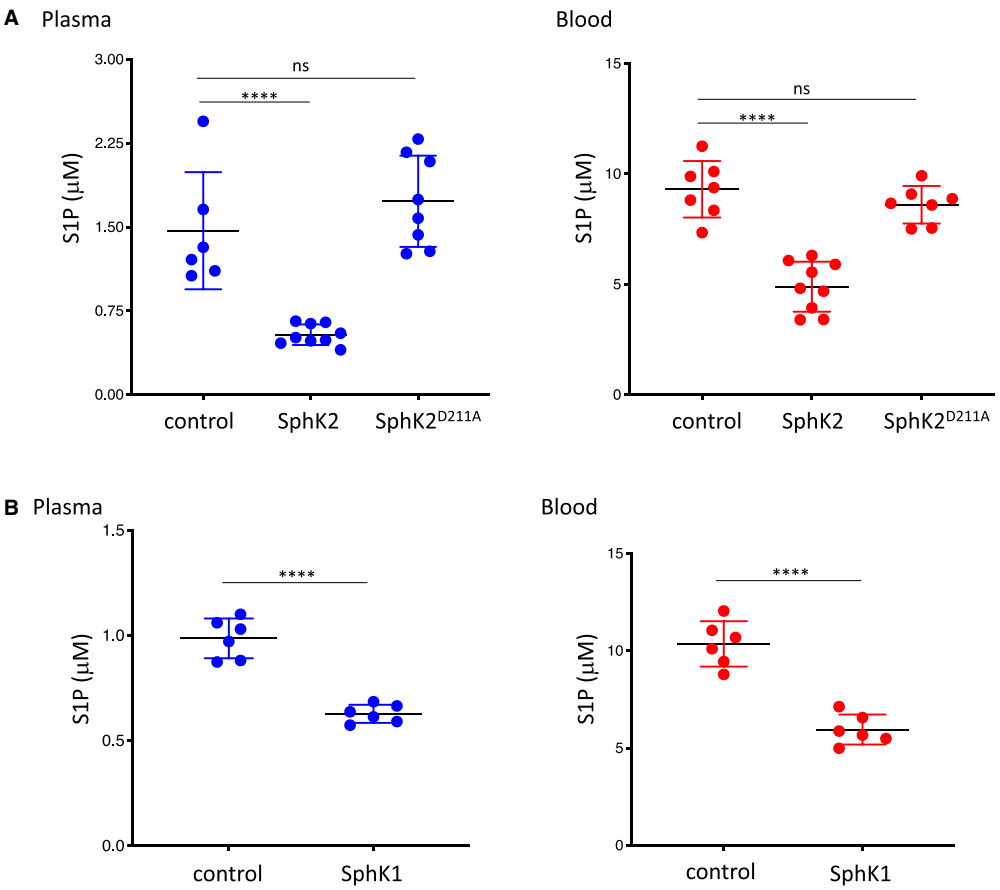

Figure 3. Circulating SIP levels in SphK2 null mice are reduced by liver Sphk expression.

Plasma and blood S1P levels of SphK2 null mice with control adenovirus or adenovirus encoding SphK2 or catalytically inactive SphK2(D211A) (**A**) or SphK1 (**B**). Plasma and blood S1P levels from C57BL/6 wild-type mice after injection of adenovirus encoding SphK1 or SphK2. Mice were bled 3 days after virus injection. One-way ANOVA followed by Sidak's multiple comparison tests (3**A**) or Student's *t*-test (3**B**) were used for estimating statistical significance. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Phosphorylated molecules such as S1P generally cannot access the interior of cells. Thus, we hypothesized that there exists a liver S1P phosphatase that dephosphorylates circulating S1P to yield sphingosine, which freely enters cells. The sphingosine absorbed by hepatocytes or other cells would be captured by SphK2-mediated phosphorylation to regenerate S1P. A candidate for such an enzyme is the non-selective lipid phosphatase Plpp3 (formerly Lpp3), which is a non-selective lipid phosphatase. This integral membrane protein has an exofacially oriented catalytic domain [33] and it is reported to shape S1P gradients in the thymus [34], and brain [35]. Furthermore, Plpp3 is reported to de-phosphorylate an S1P analog that is used to treat multiple sclerosis patients, fingolimod (FTY720) phosphate [36].

To determine whether hepatic Plpp3 is involved in clearing S1P from blood, we injected *Plpp3*$^{fl/fl}$ mice with adenovirus expressing Cre recombinase or control adenovirus. As illustrated in Figure 4A, we observed a prominent rise in both plasma and whole blood S1P when Cre-recombinase-expressing, but not control, adenoviruses were introduced. The efficacy of adenovirus-introduced Cre recombinase in reducing Plpp3 to below detectable levels is documented in Figure 4B. The prominent response observed with *Plpp3* deletion indicates the importance of this phosphatase in hydrolyzing S1P at the hepatocyte. Our result is in contrast with that from mice with *Plpp3* disrupted in the endothelium, which do not exhibit increased circulating S1P levels [37], suggesting that a different S1P phosphatase is used by endothelial cells. We measured sphingosine in liver extracts from mice with the aforementioned manipulations and found (Supplementary Figure S1) that deletion

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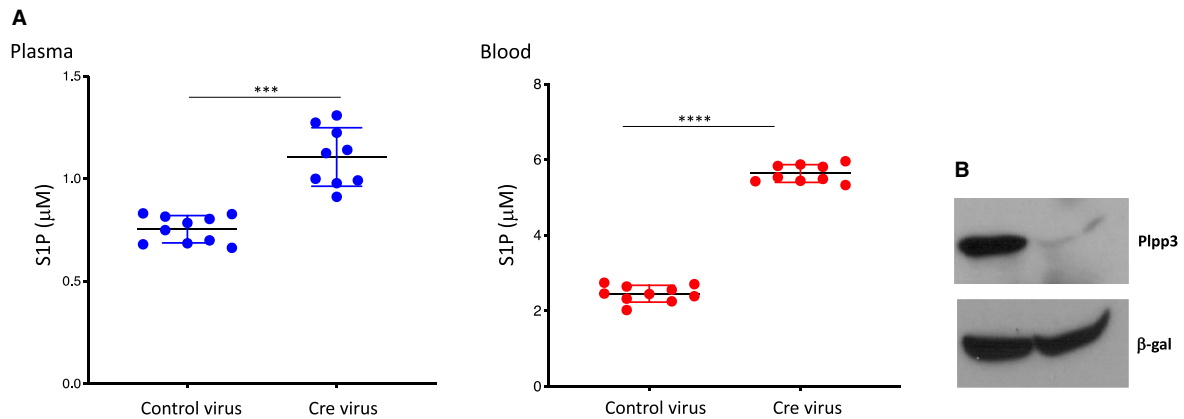

Figure 4. Elimination of liver *Plpp3* raises circulating S1P.

(**A**) Plasma (blue circles) and blood (red circles) S1P levels of *Plpp3*$^{fl/fl}$ mice following injection of control adenovirus or adenovirus encoding Cre recombinase. Student's *t*-test was used for estimating statistical significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). (**B**) Western blot of liver extract Plpp3 control (left) and cre recombinase (right) viruses probed with anti-Plpp3 sera.

of *Sphk2*, but not *Plpp3*, resulted in elevated liver sphingosine. Furthermore, the lymphocyte and red blood cells levels in the mice used in these studies were not different (Supplementary Figures S2 and S3).

Next we considered the possible fates of hepatocyte S1P, which include de-phosphorylation to sphingosine by intracellular S1P phosphatases irreversible cleavage by S1P lyase and extrusion by a S1P exporter. Given the profound disturbance of liver lipid homeostasis observed in S1P lyase null (*Sgpl*$^{-/-}$) mice [38], we tested whether deletion of the *Sgpl* gene in hepatocytes had an effect on circulating S1P levels. As depicted in Figure 5, tail vein injection of Cre recombinase, but not control, adenoviruses into *Sgpl*$^{fl/fl}$ mice resulted in a significant rise in plasma and whole blood S1P, albeit to a lesser extent than in *Plpp3*$^{fl/fl}$ mice (compare Figure 4A and Figure 5). The smaller effect of S1P lyase deficiency on blood S1P levels probably reflects the balance of intracellular S1P phosphatases, S1P export and S1P lyase in disposing the S1P captured by sphingosine kinases.

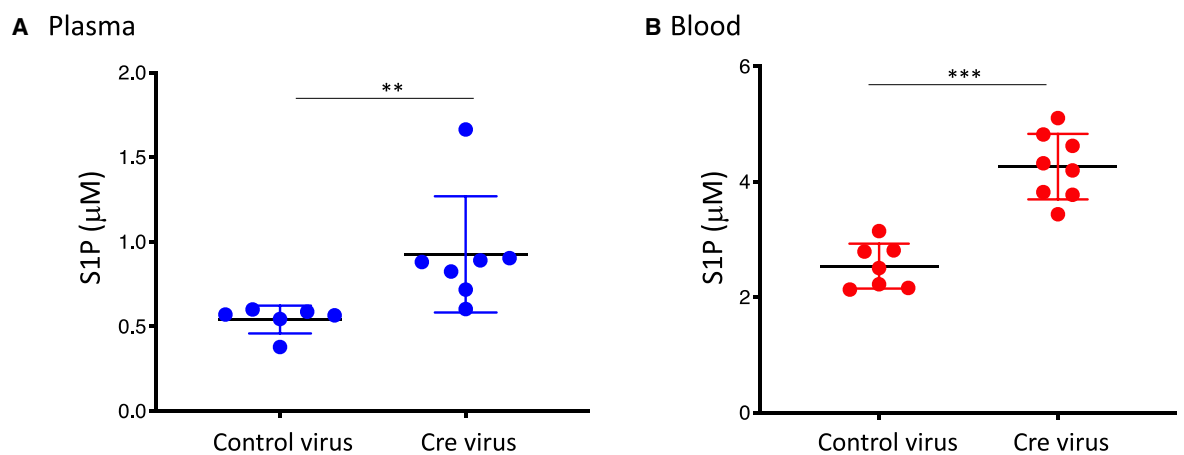

Figure 5. Plasma (blue circles) and blood (red circles) S1P levels of S1P lyase (*Sgpl*$^{fl/fl}$) mice after injection of control or cre recombinase adenoviruses.

Student's *t*-test was used for estimating statistical significance between paired groups (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

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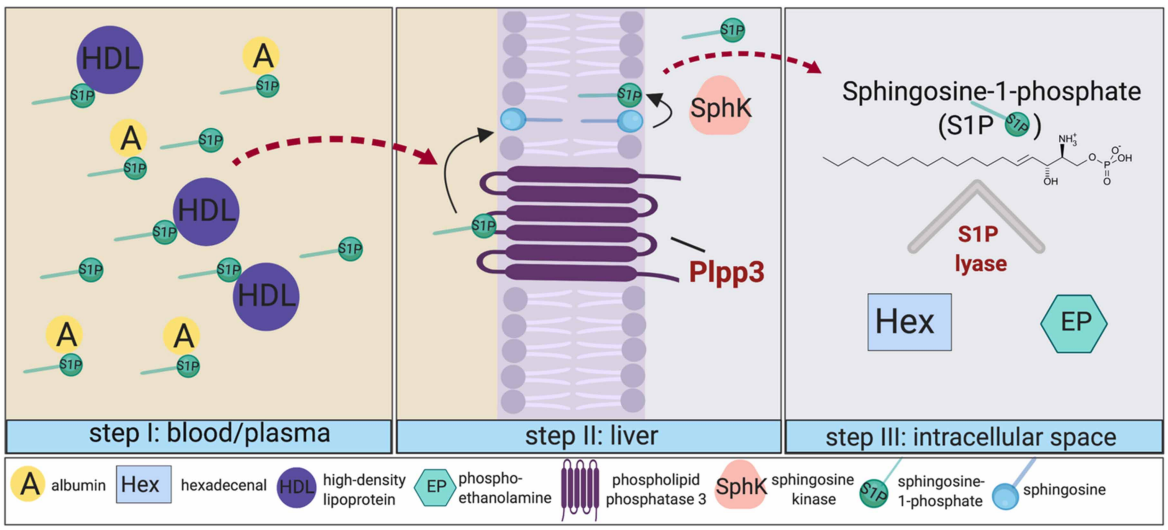

Figure 6. Illustration of mechanism whereby S1P is cleared from blood and plasma.

Discussion

The model that emerges from our results has hepatocytes, which have unfettered access to blood plasma due to the sinusoidal endothelium of the liver, as the prominent site of blood S1P disposal (summarized in Figure 6). The S1P that dissociates from plasma proteins or is released from erythrocytes (presumably via the S1P exporter Mfsd2b [39]) is hydrolyzed by the hepatocyte exofacial lipid phosphatase, Plpp3, and some of the sphingosine so formed is captured in hepatocytes by SphK-mediated phosphorylation that regenerates S1P. Because not all of the sphingosine so formed is expected to enter hepatocytes, a prediction of our model is that SphK2 inhibitors will increase circulating sphingosine as well as S1P. Indeed, this explains our observing a small rise in circulating sphingosine observed in response to SphK2 inhibitors [40]. This increased sphingosine probably serves to increase erythrocyte S1P synthesis. A final step in the hepatocyte cascade is S1P lyase activity that irreversibly degrades the S1P to form ethanolamine phosphate and a long chain aldehyde. The same model can be applied to endothelial cells, but the effect of SphK2 deficiency is less pronounced in that tissue. Although our results are largely congruent with previous reports, the observation that ligation of the liver vasculature [18,41] did not raise endogenous circulating S1P is not the result that would be anticipated from our studies. We do not have an explanation for this apparent contradiction.

The limitations of our study include a focus on mice, which is necessitated by the available genetic tools. However, we observed previously that administration of SphK1 [38] or SphK2 [19] inhibitors to rats evokes excursions in blood S1P levels similar to those observed in mice and we are unaware of a reason to suspect that the clearance of blood S1P is qualitatively different in humans or other mammals. Furthermore, our model does not afford molecular level resolution. Distinguishing among S1P clearance from different blood components, i.e. platelets and erythrocytes as well as albumin and ApoM/HDL in plasma would be valuable. In addition, the mechanism whereby S1P is transferred from erythrocytes, which are excluded from perisinusoidal space in liver, to the hepatocyte surface is unclear. A consequence of our strategy is that our study does not speak to the kinetics of S1P clearance from the various blood compartments. However, we have found that changes in whole blood S1P, which is a surrogate of the erythrocyte compartment, are detected within minutes of intravenous injection of either a SphK1 [20] or a SphK2 [19] inhibitor. Those results indicate that either the erythrocyte and plasma compartments are in rapid equilibrium or that erythrocyte S1P is somehow transferred directly to hepatocytes. Finally, our study does not address the clearance of S1P from lymph although it is the lymph S1P gradient that is necessary for proper egress of T lymphocytes into efferent lymph.

Despite such limitations, there are clear implications that emerge from our study. First, we have identified the hepatocyte as a nexus whereby an organism could, by manipulating the activity of S1P synthetic or degradative enzymes, rapidly change its circulating S1P tone, including S1P levels in erythrocytes. Second, the reduction in blood and plasma S1P levels in SphK2 null animals that we observed with forced expression of

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either SphK1 or SphK2 in hepatocytes highlights the commonality of the two enzymes. Indeed, the most meaningful difference between SphK1 and SphK2 may be tissue compartmentalization rather than the often mentioned differences in the subcellular distribution of these two isoforms. Third, engaging hepatic SphK2, Plpp3 or S1P lyase with liver-targeted inhibitors should be sufficient to drive an increase in circulating S1P, and thereby steepen the blood S1P gradient, without influence on S1P levels in extrahepatic tissues. Fourth, the high turnover rate of blood S1P affords a convenient marker of inhibition of S1P synthesis, degradation or transport, which is of practical importance in assessing inhibitors of these proteins. Finally, the model we propose provides a template for understanding the shaping of all S1P gradients.

Clinical perspective

Background: A property of mammalian, and perhaps all vertebrate, circulatory systems is high S1P concentration in blood relative to tissues. This blood — tissue S1P gradient is important both for maintaining endothelial barrier function and for the emergence of nascent lymphocytes from the thymus. S1P is cleared rapidly from blood, but the mechanism and site of clearance were previously unknown. Particularly puzzling was the role of an S1P synthetic enzyme (SphK2) in the removal of S1P from blood.

Results: Using sets of genetically modified mice, we identified the liver as the primary organ in extracting S1P from blood. Specifically, blood S1P at the hepatocyte surface is converted, via a cell surface phosphatase, to sphingosine that is absorbed and converted, via SphK2, back to S1P, which is degraded by cellular S1P lyase.

Potential Significance to human health & disease: The model that our results support predicts that the modulation of the activity of any of these liver enzymes would rapidly change circulating S1P levels. Furthermore, liver diseases could result in diminished circulating S1P.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Y.K. performed the mouse experiments, collected and analyzed data and performed statistical analysis, T.H. performed the LCMS analyses including collecting and analyzing data, A.S. generated Figure 6 and assisted Y.K. with experiments, T.E.H. designed, generated and purified the recombinant adenovirus strains, K.R.L. oversaw the mouse breeding and analyzed data, W.L.S. and K.R.L. conceived the project, Y.K. and K.R.L. wrote the manuscript; all of the authors edited the manuscript.

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Abbreviations

LCMS, Liquid Chromatography–Mass Spectrometry; MRM, multiple reaction monitoring; PBS, phosphate buffered saline; *Plpp3*, gene symbol for lipid phosphate phosphatase 3 (Lpp3); S1P, sphingosine 1-phosphate; *Sgpl*, gene symbol for S1P lyase; SphK1, sphingosine kinase 1; SphK2, sphingosine kinase 2.

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Biochemical Journal (2020) **477** 925–935
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