Mice Deficient in Sphingosine Kinase 1 Are Rendered Lymphopenic by FTY720*

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Sphingosine-1-phosphate (S1P), a lipid signaling molecule that regulates many cellular functions, is synthesized from sphingosine and ATP by the action of sphingosine kinase. Two such kinases have been identified, SPHK1 and SPHK2. To begin to investigate the physiological functions of sphingosine kinase and S1P signaling, we generated mice deficient in SPHK1. Sphk1 null mice were viable, fertile, and without any obvious abnormalities. Total SPHK activity in most Sphk1−/− tissues was substantially, but not completely, reduced indicating the presence of multiple sphingosine kinases. S1P levels in most tissues from the Sphk1−/− mice were not markedly decreased. In serum, however, there was a significant decrease in the S1P level. Although S1P signaling regulates lymphocyte trafficking, lymphocyte distribution was unaffected in lymphoid organs of Sphk1−/− mice. The immunosuppressant FTY720 was phosphorylated and elicited lymphopenia in the Sphk1 null mice showing that SPHK1 is not required for the functional activation of this sphingosine analogue drug. The results with these Sphk1 null mice reveal that some key physiologic processes that require S1P receptor signaling, such as vascular development and proper lymphocyte distribution, can occur in the absence of SPHK1.

Sphingosine-1-phosphate (S1P)† is a signaling molecule that influences cellular functions including proliferation, survival, migration, adhesion molecule expression, and morphogenesis (1–4). S1P binds to members of the S1P receptor family (also known as EDG receptors) and, via G proteins, triggers multiple signaling pathways (5, 6). S1P has also been shown to function within a conserved lipid kinase catalytic domain (15, 16). SPHK1 has a predominantly cytoplasm localization but can be phosphorylation of S1P found in circulating fluids. Since FTY720 treatment phenocopies the physiology produced by the deletion of S1P1 receptor signaling in lymphocyte trafficking was first illuminated by the activities of FTY720, a potent immunosuppressive agent. FTY720 is a sphingosine analogue that, when phosphorylated, functions as an agonist for four of the five S1P receptors (S1P1, S1P3, S1P4, and S1P5 but not S1P2) (13, 27). FTY720-P causes lymphopenia through sequestration of circulating lymphocytes within lymph nodes and Peyer’s patches and also blocks the exit of T-cells from thymus (13, 27–30). Deletion of the S1P1 receptor on thymocytes blocks egress of mature T-cells from thymus and, thus, their presence in the periphery (11, 12). Similarly T-cell trafficking of lymph nodes has been shown to depend on the presence of the S1P1 receptor on T-cells (12). In both cases, the S1P1 receptor may function to direct migration toward high levels of S1P found in circulating fluids. Since FTY720 treatment phenocopies the physiology produced by the deletion of S1P1 in lymphocytes, FTY720 may exert its effects through the interaction with the S1P1 receptor on lymphocytes with subsequent receptor inactivation. Indeed it has been shown that FTY720 may induce receptor internalization and subsequent degradation (31). FTY720 can be phosphorylated by both SPHK1 and SPHK2 in vitro (32–34), although the relevant in vivo kinase has not been identified.

We generated mice with a null Sphk1 gene to study the physiological functions of SPHK1 and its role in S1P generation. We also utilized these mice to examine the metabolism and activity of FTY720 in vivo. Our results demonstrate that...
FTY720 phosphorylation was decreased in the Sphk1 null mice, although sufficient levels of FTY720-P were generated to achieve full lymphopenia.

**EXPERIMENTAL PROCEDURES**

*Generation of Sphk1 Null Mice*—To generate the Sphk1 null mice, we cloned a genomic DNA fragment containing the Sphk1 gene from a 129/sv strain library. A 3-kb fragment containing exon 1 and exon 2 and a 7-kb fragment XhoI/NcoI that included part of exon 6 together with neomycin and thymidine kinase gene expression cassettes were used to prepare the targeting vector. The targeting vector was introduced into TC1 embryonic stem cells by electroporation, and the resulting clones were screened by Southern blotting. Targeted embryonic stem cell lines were injected into C57BL/6 blastocysts, and the chimeric male mice that were obtained were bred to C57BL/6 females. The agouti offspring were genotyped for the disrupted allele by Southern blot analysis. Heterozygous matings were set up to generate homozygous mutant mice. Mice (Sphk1−/− and littermate controls Sphk1+/− and Sphk1+/+ maintained on 129/sv × C57BL6 mixed background) were genotyped by Southern blotting using the probe shown on Fig. 1 and by PCR from tail biopsies. The primer sequences for the PCR were as follows: primer 1, 5′-TGTCACCCATGAACCTGCTGTCCCTGCACA; primer 2, 5′-AGAAGGCACTGGCTCCTCCAGAGGAACAAG; primer neo, 5′-TCGTGCTTTACGATATCGCGCCCTCCGGAT (94°C for 1 min, 65°C for 1 min, and 72°C for 1 min). The wild-type allele yielded a DNA fragment of about 300 bp, and the targeted allele yielded a fragment of ~350 bp.

**SPHK Determinations**—SPHK activity was measured essentially as described previously (35). Tissue homogenates (5–20 μg) were boiled for 3 min in 0.5 ml of 10 mM Tris-HCl, 10 mM EDTA, with 0.5 mM 4-deoxypyridoxine, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-methylumbelliferyl-β-D-glucoside, 10 μM sodium orthovanadate. Sphingosine kinase buffer: 50 mM Tris (pH 7.5), 10% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonfluoride (sodium salt), and 0.5 mM 4-deoxyxypiridine) were incubated with 50 μM sphingosine (prepared either in mixed micelles of 25 mM HCl, methanol and 1 ml of 1 M NaCl plus 100 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonfluoride, and 0.5 mM 4-methylumbelliferyl-β-D-glucoside, 10 μM sodium orthovanadate, 40 μM 4-methylumbelliferyl-β-D-glucoside, or 0.5 mM 4-methylumbelliferyl-β-D-glucoside, 10 μM sodium orthovanadate, 40 μM 4-methylumbelliferyl-β-D-glucoside). To achieve full lymphopenia, these deleted exons correspond to the 5′ and 3′ flanking regions of the protein coding sequence. For real time PCR, total RNA was purified using Trizol (Invitrogen). Mouse Sphk1, Sphk2, Sgpp1, Sgpl1, Ash2l, and Ash2h mRNAs were quantified using Assays-on-demand (Applied Biosystems, Foster City, CA) with an ABI Prism 7700 sequence detection system (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase mRNA was detected as an internal standard.

**Measurement of SIP Levels**—Lipids from tissue homogenates or serum were extracted with 1 ml of chloroform:25 mM HCl, methanol (1:1) plus 100 μl of 3 N NaOH. Organic phases were back-washed with 1 ml of 25 mM HCl, methanol and 1 ml of 1 N NaCl plus 100 μl of 3 N NaOH. For assay, the pooled aqueous phases were determined exactly as described previously (15). These deleted exons correspond to the putative lipid kinase catalytic domain, which includes the conserved domains C1, C2, C3, and part of C4 and a putative nucleotide binding motif (15, 41). Sphk1 gene-targeted embryonic stem cells were isolated and used to produce chimeric male mice, which passed on the targeted allele to their offspring. By intercrossing heterozygous mutant mice, viable homozygous Sphk1 mutant mice were obtained in the expected Mendelian ratio indicating an absence of embryonic lethality. The correct targeting of the Sphk1 locus was confirmed by Southern blotting (Fig. 1F). SacI digestion of genomic DNA and hybridization with a probe external to the region contained within the targeted vector yielded a 7-kb band corresponding to the WT allele and the predicted 5.5-kb band corresponding to the targeted allele. The homozygous Sphk1 mutant mice were fertile and lived longer than 1 year. A histologic evaluation of major organs from the Sphk1 mutant mice indicated that no abnormal pathology was present.

**RESULTS**

*Generation of Sphk1 Knock-out Mice*—The mouse Sphk1 gene is composed of six exons and five introns (Fig. 1A). To knock out Sphk1 expression in mice, a gene-targeting vector was prepared in which the region containing exons 3, 4, and 5 and the 5′-end of exon 6 was replaced by a neomycin-resistant gene cassette (Fig. 1A). These deleted exons correspond to the putative lipid kinase catalytic domain, which includes the conserved domains C1, C2, C3, and part of C4 and a putative nucleotide binding motif (15, 41). Sphk1 gene-targeted embryonic stem cells were isolated and used to produce chimeric male mice, which passed on the targeted allele to their offspring. By intercrossing heterozygous mutant mice, viable homozygous Sphk1 mutant mice were obtained in the expected Mendelian ratio indicating an absence of embryonic lethality. The correct targeting of the Sphk1 locus was confirmed by Southern blotting (Fig. 1F). SacI digestion of genomic DNA and hybridization with a probe external to the region contained within the targeted vector yielded a 7-kb band corresponding to the WT allele and the predicted 5.5-kb band corresponding to the targeted allele. The homozygous Sphk1 mutant mice were fertile and lived longer than 1 year. A histologic evaluation of major organs from the Sphk1 mutant mice indicated that no abnormal pathology was present.

To determine whether Sphk1 expression was abolished by the targeting procedure, we performed Northern blotting using RNA from kidney, the original tissue source for the purification of Sphk1 enzyme (35). An Sphk1 transcript was detected in samples from WT and heterozygote mice but not from homozygous mutant mice (Fig. 1C). To verify this result, we also performed reverse transcription-PCR using kidney RNA. No Sphk1 transcript was detected in the Sphk1−/− sample (Fig. 1D). Quantitative real time PCR was performed to measure
Sphk1 and Sphk2 mRNA expression in brain and kidney from the Sphk1−/− mice (Fig. 2A). Sphk1 mRNA was undetectable in the tissues. These results confirmed that the targeting of Sphk1 resulted in a null allele. Sphk2 mRNA levels were not altered in the Sphk1−/− mice relative to WT levels (Fig. 2A). Also we measured mRNA expression of other genes that could be involved in the S1P biosynthetic pathway (1). mRNA levels for Sgpp1, Sgpl1, Asah1, and Asah2 were not affected in the Sphk1 null brain and kidney (Fig. 2A).

Sphingosine Kinase and S1P Levels in Tissues—We next determined SPHK activity and the levels of S1P in tissues from WT and Sphk1 null mice. Enzyme assays were performed in the presence (Fig. 2B) and absence (Fig. 2C) of Triton X-100 since this detergent has been reported to stimulate SPHK1 activity and to inhibit SPHK2 activity (16). In both assays, total SPHK activity was substantially decreased relative to WT in most Sphk1 null tissues (Fig. 2, A and B), although the differences were greater when the assay was performed in the presence of Triton X-100. Lymphoid tissue, in particular, showed a large diminution in activity with a >90% reduction of SPHK activity in spleen (Fig. 2, B and C). Interestingly SPHK activity in platelets from Sphk1 null mice was not statistically different from that in WT mice (Fig. 2, B and C).

In contrast, S1P levels in most Sphk1 null tissues were similar or slightly decreased when compared with WT (Fig. 2D). An exception was serum, which normally contains relatively high levels of S1P (42). Here a substantial reduction of greater than 50% was observed in the Sphk1 null mice compared with WT (Fig. 2D, inset).

Mouse embryonic fibroblasts from Sphk1 null mice contained less than 10% of the SPHK activity detected in WT fibroblasts (Fig. 3A). The capacity of the fibroblasts to synthesize S1P was determined after incubation with sphingosine and 32P. Although both WT and Sphk1 null fibroblasts phosphorylated sphingosine to form S1P, the amount of product was about 70% lower in cells from Sphk1 null mice (Fig. 3B).

FTY720 Is Phosphorylated and Causes Lymphopenia in Sphk1 Null Mice—Signaling through the S1P1 receptor is required for the exit of lymphocytes from thymus and lymph nodes (11, 12). We analyzed the total number of lymphocytes and percentages of T- and B-cells in thymus and secondary lymph organs to determine whether lymphocyte distribution was affected in Sphk1 null mice. No significant differences were observed in cellularity or in T-cell or B-cell percentages in thymus, spleen, peripheral and mesenteric lymph nodes, Peyer’s patches (Table I), or blood (Fig. 4, A and B) from Sphk1 null mice compared with control WT.

Intravenous administration of FTY720 was equally effective in reducing blood lymphocytes in both Sphk1 null and WT mice even at the lower dose used (Fig. 4, A and B). At 3 mg/kg FTY720, total lymphocyte numbers were reduced an average of 64% (p < 0.05) with T-cells lowered by 85% (p < 0.01) and B-cells lowered by 53% (p < 0.05). When 0.3 mg/kg FTY720 was used, the total lymphocyte numbers decreased about 78% (p < 0.01), the T-cell numbers decreased 92% (p < 0.01), and B-cell numbers decreased 77% (p < 0.01). Phosphorylation of FTY720 is required for altering lymphocyte trafficking (13, 27), and liquid chromatography-mass spectrometry analysis revealed the presence of phosphorylated FTY720 (FTY720-P) (Fig. 4, C and D) in the two groups at both doses analyzed. FTY720-P levels in Sphk1 null mice treated with 3 mg/kg and 0.3 mg/kg FTY720 were 41% (p < 0.05) and 24% (p < 0.12) lower, respectively, than in WT. In both cases, absolute levels of FTY720-P were sufficient to have achieved full lymphopenia (Fig. 4).

Plasma S1P was reduced 65% (p < 0.001) in Sphk1 null mice. At the doses used in this study, FTY720 appeared not to alter...
circulating plasma S1P levels in WT (Fig. 4, C and D). The lower S1P levels in plasma relative to serum (Fig. 2D, inset) are consistent with S1P release by platelets during blood clotting (42, 43).

**DISCUSSION**

This is the first report of a knock-out for a sphingosine kinase gene in mammals. Mice null for the Sphk1 gene are viable and are, thus, distinct from S1P1 receptor null mice, which die in utero due to a block in vascular system development (9). The strikingly different phenotype between these two knock-out mice indicates that S1P signaling was not abolished in Sphk1 null mice. Indeed, with the deletion of the Sphk1 gene, SPHK catalytic activity was substantially but not completely reduced in most tissues. The remaining SPHK activity in Sphk1 null tissues was likely to be contributed, at least in part, by SPHK2.

**Table 1**

| Tissue  | Total cells (×10^6) | Percentage of T-lymphocytes | Percentage of B-lymphocytes |
|---------|---------------------|------------------------------|------------------------------|
|         | Sphk1+/+            | Sphk1+/-                       | Sphk1+/+                      | Sphk1+/-                       |
| Thymus  | 21 ± 2              | 89 ± 2                        | 88 ± 6                       | 43 ± 11                       | 44 ± 6                       |
| Spleen  | 25 ± 2              | 43 ± 9                        | 44 ± 3                       | 31 ± 5                        | 32 ± 4                       |
| PLN     | 6 ± 2               | 67 ± 16                       | 60 ± 8                       | 63 ± 3                        | 65 ± 2                       |
| MLN     | 4 ± 1               | 63 ± 3                        | 65 ± 2                       | 28 ± 3                        | 25 ± 6                       |
| PP      | 2 ± 1               | 63 ± 3                        | 65 ± 2                       | 28 ± 3                        | 25 ± 6                       |

The total number of cells was determined by counting isolated cells using a Coulter counter. The percentages of T- and B-lymphocytes were calculated by flow cytometry using phycoerythrin-conjugated CD3ε and fluorescein isothiocyanate-conjugated B220 antibodies, respectively. Values represent means ± S.D., n = 4 for total cell numbers and thymus and spleen determinations, n = 3 for peripheral lymph node and n = 2 for mesenteric lymph node and Peyer’s patch determinations. PLN, peripheral lymph nodes; MLN, mesenteric lymph nodes; PP, Peyer’s patches.
FIG. 4. FTY720 causes lymphopenia in Sphk1 null mice. A and B, lymphocyte counts in blood after treatment with 3 mg/kg (A) or 0.3 mg/kg FTY720 (B). Blood lymphocyte and T- and B-cell counts were determined by flow cytometry. Results are expressed as the mean ± S.D., n = 3. Statistical significance was calculated by t test (*, p < 0.05; **, p < 0.005 for FTY720-treated versus vehicle-treated groups). C and D, phosphorylation of FTY720 in vivo. Sphk1 null and WT mice were treated with 3 mg/kg (C) or 0.3 mg/kg FTY720 (D). Plasma levels of S1P (open bars), FTY720 (gray bars), and FTY720-P (black bars) were determined as described under “Experiment Procedures.” Results are expressed as the mean ± S.D., n = 6. Statistical significance was calculated by t test (*, p < 0.05; **, p < 0.005 for Sphk1−/− versus WT mice).

which has been found to be widely distributed among mouse tissues (16, 26). Additional SPHKs are possible, but others have not been identified.

Although our results indicate that the Sphk1 gene contributes the major SPHK activity in most of the tissues analyzed, levels of the product, S1P, were not substantially reduced in the corresponding Sphk1 null tissues. This may reflect compensatory mechanisms to maintain critical cellular S1P levels and likely explains the lack of a severe phenotype. In addition to SPHK2 and possibly other kinases, autotaxin (lysophospholipase D) may also be a source of extracellular S1P generation via hydrolysis of sphingosylphosphorylcholine (44, 45). Also changes in the activities of S1P lyase, S1P phosphatase, or ceramidases might contribute to the maintenance of the S1P levels in Sphk1 null mice. However, no changes in mRNA expression of these genes, Sgppl, Sgpfl, Asah1, and Asah2, were detected after Sphk1 deletion.

Serum and plasma levels of S1P in the Sphk1 null mice were reduced to less than 50% of WT, indicating that SPHK1 has an important but not an exclusive role in regulating serum levels of S1P. Even with this reduction in the Sphk1 null mice, the absolute S1P levels in serum and plasma were in the micromolar range, although the actual amount of S1P that is bioavailable is not known. Our determinations of plasma and serum S1P concentrations in mouse are higher than the reported human levels (42, 46).

The lymphocyte S1P1 receptor controls egress of mature thymocytes from the thymus and of lymphocytes from lymph nodes (11, 12). Circulating levels of S1P, which could act on the S1P1 receptor as a migratory signal, may be key to these trafficking events. We did not find alterations in cellularity of lymphoid organs or in distribution of T- and B-cells between lymphoid compartments. These results indicate that if circulating levels of S1P are critical for lymphocyte trafficking, the lowered levels in serum and plasma in the Sphk1 null mice are sufficient to maintain this process.

Previous in vitro studies have demonstrated that the sphingosine analogue FTY720 can be a substrate for both SPHK1 and SPHK2 (32–34). Although some studies had indicated that SPHK2 was more efficient than SPHK1 in catalyzing FTY720 phosphorylation, the enzyme responsible for the in vivo modification of FTY720 was not known. Factors such as accessibility of the substrate to cellular compartments and tissue distribution of the enzymes could influence FTY720 phosphorylation. In the Sphk1 null mice, administered FTY720 was converted to FTY720-P about 25–40% less efficiently than in WT mice, depending on the dose used. The reduction of FTY720-P production in Sphk1 null mice may indicate that SPHK1 normally acts on FTY720 in vivo or may be due to an adaptive shift in equilibrium of competing substrate pools in Sphk1 null mice. However, sufficient levels of FTY720-P were reached in the Sphk1 null mice to generate lymphopenia. These results demonstrate that SPHK1 is dispensable for the functional expression of an immunosuppressive response to FTY720. Although we cannot rule out possible contributions by other unknown kinase activities, these results are consistent with the conclusion that SPHK2 is sufficient for FTY720 functional activation in vivo.

In summary, we have reported the first genetic deletion of Sphk1 in mice. Although S1P receptor signaling is essential for lymphocyte trafficking and development of the vascular system in mice, there was no evidence that Sphk1 null mice have major abnormalities in these processes. Severe disruption of physiologic S1P signaling is likely to require substantially reducing circulating and tissue levels of S1P by the simultaneous deletion of multiple SPHK genes. Finally the Sphk1 null mice will be valuable for exploring the role of SPHK1 in vascular function, cancer, autoimmunity, and infertility (47).

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