Normal acute and chronic inflammatory responses in sphingosine kinase 1 knockout mice

Jason Michauda, Masataka Kohnob, Richard L. Proiac, Timothy Hlaa,*

a Center for Vascular Biology, Department of Cell Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3501, USA
b Inflammation and Immunology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto 602-8566, Japan
c Genetics of Development and Disease Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

Received 22 May 2006; revised 5 July 2006; accepted 10 July 2006
Available online 21 July 2006
Edited by Sandro Sonnino

Abstract Sphingosine-1-phosphate, generated from the phosphorylation of sphingosine by sphingosine kinase enzymes, is suggested to function as an intracellular second messenger for inflammatory mediators, including formyl peptide, C5a, and Fc. More recently, a role for sphingosine kinases during inflammation has also been proposed. Here we show that sphingosine kinase 1 knockout mice exhibit normal inflammatory cell recruitment during thioglycollate-induced peritonitis and that sphingosine kinase 1-null neutrophils respond normally to formyl peptide. In the collagen-induced arthritis model of rheumatoid arthritis, sphingosine kinase 1 knockout mice developed arthritis with normal incidence and severity. Our findings show that sphingosine kinase 1 is dispensable for inflammatory responses and support the need for more extensive studies of sphingosine kinases in inflammation.
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Sphingosine kinase; Sphingolipids; Inflammation; Arthritis

1. Introduction

The lysophospholipid sphingosine-1-phosphate (S1P) acts as an extracellular ligand at S1P receptors to regulate diverse physiologic functions, including angiogenesis, lymphocyte recirculation, vascular permeability, and heart rate [1–3]. S1P is also suggested to function as an intracellular second messenger in mobilizing calcium, promoting cell proliferation and survival [4]. In addition, S1P is a critical intermediate in sphingolipid and glycerophospholipid metabolism. It is generated from the phosphorylation of sphingosine by sphingosine kinase enzymes (Sphk1 and Sphk2) and is degraded by S1P phosphatases (Spp1 and Spp2) and S1P lyase (SPL). Thus, Sphks are fundamental to regulating S1P levels and its intracellular roles.

Recently, the role of Sphk in immunity and inflammation has been the focus of intense study. In mast cells, macrophages, and neutrophils, Sphk activity is stimulated by the ligation of plasma membrane receptors for C5a, Fc and formyl peptide (MLP) [5–8]. Inhibition of Sphk activity and anti-sense knockdown of Sphk1 inhibited C5a-stimulated Ca2+ mobilization, enzyme release, and chemotaxis in both neutrophils and macrophages [7,8]. In addition, experimental models using pharmacologic inhibitors support a role for Sphk in acute inflammatory states. Pretreatment of mice with a Sphk inhibitor, dimethylsphingosine (DMS), prevented C5a-induced peritonitis [9]. However, DMS is known to have non-specific, off-target effects [10,11]. Inhibition of Sphk was also shown to prevent neutrophil activation and lung permeability in a model of trauma and hemorrhagic shock [12]. To date, the effects of Sphk inhibition on inflammation have been attributed to intracellular S1P-mediated signaling. Thus, previous work suggests a role for Sphk during inflammation, although the significance in vivo remains unclear.

In this study, we sought to determine if Sphk1 is essential during inflammation using Sphk1 knockout mice [13]. We examined the requirement for Sphk1 in the acute inflammatory responses that occur in thioglycollate-induced peritonitis. We also analyzed the function of Sphk1 during chronic inflammation using the collagen-induced arthritis (CIA) model of rheumatoid arthritis (RA), which shares clinical, immunological, and histological features with the human disease of RA [14].

2. Materials and methods

2.1. Mice

Sphk1 null mice (Sphk1–/-) were generated and genotyped as described [13]. For neutrophil and peritonitis experiments, mice were backcrossed five generations to C57BL/6 (Jackson Laboratory). All mice were housed under specific pathogen-free conditions. Animal care and experimental procedures were approved by the University of Connecticut Animal Care Committee.

2.2. Thioglycollate-induced peritonitis

8–12 week-old male Sphk1–/- and Sphk1+/+ mice were injected intraperitoneally (IP) with 2 ml sterile thioglycollate (TG) (3% wt/vol). Four hours after challenge, mice were lavaged with 5 ml cold Hanks buffered saline (HBSS, Invitrogen). Total leukocyte counts were determined using a Coulter counter (Beckman Coulter) and manual leukocyte differentials were counted from three high-power fields of May–Grunwald Giemsa (Sigma) stained cytospin preparations.

Abbreviations: S1P, Sphingosine-1-phosphate; Sphk1, Sphingosine kinase 1; Spp, Sphingosine-1-phosphate phosphatases; SPL, Sphingosine-1-phosphate lyase; CIA, Collagen-induced arthritis
2.3. Neutrophil studies

Thioglycollate-elicited neutrophils were isolated by peritoneal lavage with 5 ml cold HBSS four hours after I.P. challenge with 2 ml thioglycollate (3% wt/vol). Cells were >80% neutrophils by May-Grunwald Giemsa staining, and >95% viable by trypan blue exclusion. N-formyl-Met-Leu-Phe (fMLP) (Sigma) was dissolved in DMSO and stored as single-use aliquots at -80 °C. NADPH oxidase activity was measured using superoxide dismutase-inhibitable isoluminol chemiluminescence [15]. 5 × 10^6 cells were suspended in 50 μM isoluminol (Sigma), 5 U/ml horseradish peroxidase (MP Biomedical), in phosphate buffered saline with 0.5 mM CaCl_2, 0.5 mM MgCl_2, and 7.5 mM d-glucose (PBSG). Cell suspensions were added to a 96 well plate, stimulated with 1 μM fMLP or PBSG, and read on a luminescence plate reader over 5 min. For immunoblot analyses, 2 × 10^6 neutrophils in PBSG were stimulated with 1 μM fMLP at 37 °C for indicated times, lysed in 6x sample buffer (at 1x: 50 mM Tris, pH 7.5, 1.5% SDS, 0.2% deoxycholate, 0.2% Triton X-100, 10 mM MgCl_2), and DNA was sheared by passing through a 25G needle 5–10 times. Equal amounts of protein were separated by 10% SDS/PAGE and blotted onto a nitrocellulose membrane. Immunoblot analyses were performed using phospho-specific antibodies for ERK p42/44, p38, AKT (Cell Signaling) and pPAK (SantaCruz). mRNA expression analysis, RNA extraction, cDNA synthesis and real-time PCR analysis were performed as described [16]. Briefly, total RNA was extracted from elicited neutrophils using RNA-STAT-60 (Tel-Test) following manufacturer’s instructions. RNA was treated with DNase I, followed by reverse transcription. Equal amounts of cDNA were run in duplicate SYBR green PCRs using an ABI 7900HT sequence detection system (Applied Biosystems). Primer sets for PCR were Sphk1 (5'-GGAGGAGGCGAGATACCCCTCTGAACTGCGCA-3', 5'-GATGGTGATAGTTAGGG-3'), Sphk2 (5'-GCCCGAGATTTGCTCTATGCT-3', 5'-GATGGTGATAGTTAGGG-3'), Spp1 (5'-GGGGTCTGTGTCATGATCCTG-3', 5'-GAGAAGGAGATGCTTCATGGGC-3'), Spp2 (5'-GTCCTGTTGTCATGGGCC-3', 5'-GGAGGGAGGAGGAGGAGGAG-3'), SPL (5'-GCCGAGATTTGCTCTATGCT-3', 5'-CTGGTATGAGTTAGGG-3'), b-actin (5'-GGAGGAGGAGGAGGAGGAGGAGGAGGAG-3'). Data were analyzed to account for reaction efficiency [17] and results expressed relative to b-actin expression.

Fig. 1. Sphk1+/− mice develop peritonitis in response to TG. Sphk1+/+ and Sphk1−/− mice (n = 9 per group) were injected i.p. with TG and 4 h later cells were collected by peritoneal lavage. (A) Total cell number and (B) leukocyte composition of cells recruited to the peritoneum. Data shown are the means ± S.E. No statistically significant differences were observed in (A) (P = 0.234), or (B).

Fig. 2. fMLP-induced NADPH oxidase activity and fMLP receptor signaling does not require Sphk1. (A) Superoxide generation by Sphk1+/+ and Sphk1−/− neutrophils stimulated with 1μM fMLP. Results are represented in relative light units (RLU). (B) Kinase phosphorylation in Sphk1+/+ and Sphk1−/− neutrophils stimulated with 1μM fMLP. (C) Neutrophil Sphk1 and Sphk2 mRNA levels. (D) Neutrophil Spp1, Spp2 and SPL mRNA levels. Data shown are the means ± S.D. of at least two independent experiments.
2.4. Induction and assessment of collagen-induced arthritis

Sphk1 mice were backcrossed 6 generations to DBA/1 mice (Jackson Laboratory). CIA was induced on day 1 in 6–7 week-old male Sphk1−/− and Sphk1+/+ mice by intradermal tail base injection of 100 µg bovine collagen II (CII) supplemented with 2.0 mg/ml M. tuberculosis (Chondrex) emulsified in complete Freund’s adjuvant (CFA) [18]. On day 21, mice received an intradermal tail base injection of 100 µg CII in incomplete Freund’s adjuvant. Clinical severity of disease was evaluated by a blinded examiner every 4 days [19]. Each paw was scored for inflammation on a scale of 0–4: 0, normal; 1, erythema and mild swelling confined to ankle, or tarsals, or individual digits; 2, moderate erythema and swelling of tarsals and ankle; 3, severe erythema and mild swelling of ankle, tarsals and digits; 4, severe erythema and severe swelling of ankle, tarsals and digits. Total daily scores for each mouse were obtained by adding scores from all four paws. On day 60 mice were euthanized and forepaws were weighed. For histology, paws were fixed with 10% formalin, decalcified in Decal (Fisher), embedded in paraffin, and 5 µm sections were stained with hematoxylin/eosin.

2.5. Measurement of S1P levels

Peritoneal exudates were collected, centrifuged at 500 × g for 5 min, and supernatants stored at −80°C. For determining S1P levels during arthritis, paws were severed at the wrist joint and tissues digested with 0.4 mg proteinase K in 0.3 ml of 1 mM Tris, pH 8.5, at 60°C for 2 h, sonicated on ice, centrifuged, and supernatants stored at −80°C. S1P levels were analyzed by HPLC with a fluorescent detection system (Shimadzu) after lipid extraction and derivatization with o-phthalaldehyde as previously described [20].

2.6. Data analysis

Results are representative of at least three independent experiments. Statistical significance was determined by unpaired Student’s t test using P < 0.05 as significant.

3. Results

3.1. Sphk1 knockout mice exhibit normal inflammatory cell recruitment

Acute inflammation is characterized by the rapid recruitment of leukocytes from the periphery to the site of inflammation. To analyze the role of Sphk1 in acute inflammation, peritonitis was induced in Sphk1−/− and Sphk1+/+ mice by thioglycollate injection [21]. In both Sphk1−/− and Sphk1+/+ mice, inflammatory cells were recruited into the peritoneum at 4 h post-injection (Fig. 1A), and maintained equivalent cell numbers through at least 48 h (data not shown). There was no significant difference in the composition of cells (as determined by differential cell counts) obtained from Sphk1−/− and Sphk1+/+ mice, with granulocytes being predominant in both (Fig. 1B). Sphk1−/− mice show a greater than 50% reduction in serum S1P levels, but relatively equivalent S1P levels in other tissues [13]. Similarly, peritoneal exudates in Sphk1−/− and Sphk1+/+ mice with thioglycollate-induced peritonitis contained low levels of S1P (19–20 nM), with no significant differences between wild-type and Sphk1−/− groups. This suggests that tissue S1P levels at the inflammatory site are low compared to plasma levels (~400 nM) and that local action of Sphk2 is sufficient to compensate for the lack of Sphk1.

3.2. NADPH oxidase activity and fMLP signaling

Neutrophils are often the primary cell type recruited during acute inflammation. Once activated, they display a variety of microbicidal responses including phagokinetic activity and superoxide generation. We therefore examined the ability of Sphk1−/− and Sphk1+/+ neutrophils to produce superoxide. No difference in superoxide production in response to fMLP was evident in neutrophils lacking Sphk1 (Fig. 2A). To confirm that fMLP-induced signaling was normal in Sphk1−/− neutrophils, we determined the activation levels of several kinases downstream from the fMLP receptor. As shown in Fig. 2B, fMLP induced equivalent phosphorylation of p38 mitogen-activated kinase (p38), p44/42 mitogen-activated kinase (ERK), protein kinase B (Akt), and p21-activated kinase (PAK) in Sphk1−/− and Sphk1+/+ neutrophils.

3.3. Sphk expression levels in Sphk1−/− neutrophils

In order to rule out possible upregulation of Sphk2 in Sphk1−/− mice, we determined the mRNA levels of both known Sphk isoforms using real-time PCR. Sphk2 mRNA levels were not elevated in Sphk1−/− neutrophils, which as expected, lacked Sphk1 mRNA (Fig. 2C). In addition, mRNA levels of other S1P-metabolizing enzymes, including S1P phosphatases (Spp1 and Spp2) and S1P lyase (SPL) were not altered in Sphk1−/− neutrophils (Fig. 2D). The absence of compensatory Sphk2 upregulation in Sphk1−/− mice is consistent with previous studies [13].
3.4. Sphk1 knockout mice are susceptible to CIA

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovitis. Recently, Sphk1 was proposed as a regulator of B cell apoptosis in RA [22]. We assessed the possible involvement of Sphk1 in RA using the murine CIA model[18]. This well established model induces T-cell dependent autoimmunity, resulting in chronic synovitis and joint destruction [23]. Loss of Sphk1 did not affect the development or progression of CIA, as Sphk1−/− and Sphk1+/+ mice developed severe joint inflammation of the hind and forepaws, as assessed by clinical scores (Fig. 3A). The incidence of CIA in both groups reached 100% 40 d after immunization, with >2 fold increase in forepaw weight on day 60 (Fig. 3B). Histopathologic analysis confirmed the development of CIA. 60 d after immunization, Sphk1−/− and Sphk1+/+ mice exhibited CIA-characteristic chronic synovial inflammation, pannus formation and cartilage destruction (Fig. 4). Tissue levels of S1P were also measured. S1P levels in forepaws of naive Sphk1−/− and Sphk1+/+ mice were 5.5 ± 0.4 and 3.9 ± 0.5 pmol/mg protein. No change in S1P levels were seen with the induction of CIA (5.2 ± 1.3 and 4.9 ± 0.7 pmol/mg protein, Sphk1−/− and Sphk1+/+, respectively). This suggests that compensation by the Sphk2 enzyme occurred in the inflamed joints of Sphk1−/− mice.

4. Discussion

Sphk1 has been suggested to function as a second messenger in a variety of immune cells, including neutrophils, mast cells, and macrophages [5–8]. In vivo studies with pharmacologic Sphk inhibitors further support a role for Sphks in immune cell function [9,12]. With these observations in mind, we expected that mice lacking Sphk1 would have defective inflammatory responses. In this study we have shown that Sphk1 is not required for inflammatory cell recruitment during thioglycollate-induced peritonitis. Compared to wild-type controls, Sphk1−/− mice were able to recruit equivalent numbers of neutrophils and monocytes/macrophages to the peritoneal cavity. This suggests that the overall inflammatory cascade and resulting migratory response of leukocytes were not significantly altered by the absence of Sphk1. We have also shown that Sphk1 null neutrophils generate large amounts of superoxide in response to fMLP, and that kinase signaling events downstream of the fMLP receptor remain intact. Although we cannot rule out signaling functions of Sphk1 during the action of proinflammatory ligands other than fMLP, there is no gross deficit during acute inflammation in the Sphk1 knockout mouse.

Sphk1 null mice were also able to develop chronic inflammation, showing prominent joint destruction in the CIA model of
RA. CIA is pathogenically an autoimmune disease involving anti-CII antibodies and adaptive immunity [24]. T and B cells are critical for disease initiation, with other inflammatory cells recruited to affected joints. Thus, the ability of Sphk1 knock-out mice to develop CIA suggests that T and B cells do not rely on Sphk1 in order to function normally in generating adaptive immune responses.

SIP function as an inflammatory mediator in the extracellular milieu is an attractive idea that draws parallels to prostanooids and other lipid mediators. Although blood contains large stores of S1P, tissue levels are generally much lower; which is likely the result of increased degradation. Indeed, we failed to detect any increase in tissue SIP levels during acute or chronic inflammatory states. This questions the role of SIP as an inflammatory mediator, but does not negate possible micro-environment or intracellular functions.

To fully understand our results, it is important to distinguish our study, using Sphk1 knockout mice, with others using pharmacological inhibitors. Most studies of Sphks to date have used sphingolipids to competitively inhibit Sphk activity [5,7,9]. Such inhibitors, including dihydrosphingosine (DHS) and dimethylsphingosine (DMS), are known to inhibit protein kinase C (PKC) as well [10,11]. Indeed, their properties as neutrophil inhibitors have been attributed to PKC inhibition [25–28]. Thus when using sphingolipid inhibitors it is difficult to separate effects on PKC from those on Sphk. In addition, sphingosine and derivatives also profoundly alter ion channel activity [29]. The exception may be the Sphk inhibitor SKI-2, whose specificity for Sphk is purportedly higher [30]. Moreover, all of the current Sphk inhibitors are isoform-non-specific, i.e. inhibiting both Sphk1 and Sphk2.

In contrast, by disrupting the Sphk1 gene we have in effect wholly and singly inhibited the Sphk1 enzyme. Although previous studies have targeted Sphk1 with antisense knockdown [7,8,31], the effects of long-term and global loss of Sphk1 remain unknown. To this end, we have confirmed that Sphk2 is not upregulated after the loss of Sphk1 [13]. These data show that Sphk1 activity is dispensable for inflammatory responses under the conditions examined here. This suggests that the functions of Sphk1 and Sphk2 may be redundant. Alternatively, it is possible that mice lacking the Sphk1 gene during embryonic development may be adapted to not rely on this pathway for inflammatory responses postnatally. Thus, acute inhibition of Sphk in normal animals may well lead to modulation of inflammatory responses. However, the normal phenotype of Sphk1 null mice during acute and chronic inflammation supports the need for more extensive studies of Sphks in immune function.

Acknowledgments: We thank Nancy Ryan for assistance with histochemical procedures, and the lab of Dr. Dianqing Wu for assistance in experimental procedures. This work was supported by NIH Grants HL70694 and HL67330 (T.H.). J.M. is supported by the National Institutes of Health Medical Scientist Training Program.

References


