SphK1 mediates hepatic inflammation in a mouse model of NASH induced by high saturated fat feeding and initiates proinflammatory signaling in hepatocytes

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Abstract Steatohepatitis occurs in up to 20% of patients with fatty liver disease and leads to its primary disease outcomes, including fibrosis, cirrhosis, and increased risk of hepatocellular carcinoma. Mechanisms that mediate this inflammation are of major interest. We previously showed that overload of saturated fatty acids, such as that which occurs with metabolic syndrome, induced sphingosine kinase 1 (SphK1), an enzyme that generates sphingosine-1-phosphate (S1P). While data suggest beneficial roles for S1P in some contexts, we hypothesized that it may promote hepatic inflammation in the context of obesity. Consistent with this, we observed 2-fold elevation of this enzyme in livers from humans with nonalcoholic fatty liver disease and also in mice with high saturated fat feeding, which recapitulated the human disease. Mice exhibited activation of NFκB, elevated cytokine production, and immune cell infiltration. Importantly, SphK1-null mice were protected from these outcomes. Studies in cultured cells demonstrated saturated fatty acid induction of SphK1 message, protein, and activity, and also a requirement of the enzyme for NFκB signaling and increased MCP1 mRNA encoding TNFα and MCP1. Moreover, saturated fat-induced NFκB signaling and elevation of TNFα and MCP1 mRNA in HepG2 cells was blocked by targeted knockdown of S1P receptor 1, supporting a role for this lipid signaling pathway in inflammation in nonalcoholic fatty liver disease.—Geng, T., A. Sutter, M. D. Harland, B. A. Law, J. S. Ross, D. Lewin, A. Palanisamy, S. B. Russo, K. D. Chavin, and L. A. Cowart. SphK1 mediates hepatic inflammation in a mouse model of NASH induced by high saturated fat feeding and initiates proinflammatory signaling in hepatocytes. J. Lipid Res. 2015. 56: 2359–2371.

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Nonalcoholic fatty liver disease (NAFLD) has increased dramatically in recent years due to the rising incidence of obesity and type 2 diabetes (1). NAFLD occurs as a continuum of pathological changes in the liver initiated by hepatic steatosis, which, in up to 20% of individuals, may progress to hepatic inflammation, cirrhosis, and/or cancer (2). Although this progression constitutes a critical switch from a benign to a harmful status, the underlying molecular mechanisms are poorly understood.

The dyslipidemia that occurs in the metabolic syndrome (of which fatty liver disease is considered the hepatic manifestation) overloads tissues with lipids, leading to toxic effects such as apoptosis, insulin resistance, maladaptive autophagy, and endoplasmic reticulum (ER) stress (3); these processes are collectively referred to as “lipotoxicity.” Lipotoxic processes are thought to mediate, at least in part, organ-specific disease processes that ensue in the obese context (4, 5). A major mechanism linking lipid oversupply to pathophysiological consequences of obesity is through perturbed synthesis of bioactive lipids. For example, studies by others and us have demonstrated roles for the aberrant production of bioactive sphingolipids in processes including skeletal muscle insulin resistance (6, 7), pancreatic β-cell death (8, 9), development of atherosclerotic lesions (10), diabetic cardiomyopathy (11), and many other undesirable outcomes of obesity (12). Perturbation of lipid biosynthesis occurs, in part, through oversupply of substrates for sphingolipid biosynthetic enzymes.

Abbreviations: CD, control diet; ER, endoplasmic reticulum; HPF, high-powered field; HSFD, high saturated fat diet; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ORO, Oil Red O; SphK1, sphingosine kinase 1; S1P, sphingosine-1-phosphate; S1P1(2), sphingosine-1-phosphate receptor 1(2).

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but we have also shown roles for saturated fatty acids in increasing enzymes of sphingolipid metabolism. For example, we previously observed that lipid oversupply (as occurs in metabolic disease, the physiological context of NAFLD) induced sphingosine kinase 1 (SphK1) (7, 13), a finding that has since been corroborated by other groups (14–16). These studies were the first to demonstrate fatty acid-mediated induction of SphK1 transcription, and perhaps more importantly, placed SphK1 in the context of cell lipotoxicity and metabolic disease.

Roles of SphK1 in liver have been addressed by only a handful of studies in diverse disease models, many of which argue for a beneficial role of the enzyme. For example, SphK1 protected against ethanol-induced hepatocyte injury and also from bile salt-induced apoptosis (17, 18). Moreover, some data suggested that SphK1 played a role in insulin sensitization by adiponectin (19). However, because our recent studies indicated that saturated fatty acids induced SphK1 expression in isolated skeletal muscle myotubes and that SphK1 increased IL-6 in primary skeletal muscle cells and also in skeletal muscle in vivo (13), we sought to test whether SphK1 might increase in liver under conditions of high saturated fat feeding and, if so, to test its potential contribution to proinflammatory signaling NAFLD. Therefore, the purpose of this study was to test whether SphK1 increases in NAFLD, whether it plays a proinflammatory role, and, if so, to determine the mechanism.

MATERIALS AND METHODS

Experimental animals

Six-week-old male C57BL/6J (Jackson Laboratories, Bar Harbor, ME) and SphK1−/− mice (20) in the C57BL/6J background (originally obtained from Dr. Richard Proia, National Institute of Diabetes and Digestive and Kidney Disease, and maintained by the Center of Biomedical Research Excellence in Lipidomics and Pathobiology Animal Core at the Medical University of South Carolina) were housed in groups of three to four per cage in a light- (12:12 h light-dark cycle) and temperature-controlled quarters (21°C) and were provided with water and normal chow (#5001 PMI Nutrition, Brentwood, MO) ad libitum. Mice were split into groups of seven to eight (in two cages per group) and were provided test and control diets (CDs) ad libitum after 2 weeks of acclimation. The diets were purchased from Harlan Laboratories, Inc. (Indianapolis, IN), including a custom milkfat-based high-fat diet (TD.09766, in which 60% of the total calories were derived from milkfat; for exact composition refer to supplementary Table 1), and a standard isocaloric low-fat diet (TD.08810, in which 10% of the total calories were derived from fat). Mice were euthanized humanely by isoflurane (Hospira, Inc., Lake Forest, IL) followed by cervical dislocation after 18 weeks of feeding. All experiments were conducted in accordance with University regulations.

Collection of mouse tissue and sera

The peripheral blood samples were collected from mice under isoflurane inhalation anesthesia by sterile cardiac puncture. Mice were then euthanized humanely by isoflurane and cervical dislocation. Livers were promptly dissected from the animals and weighed after gallbladder removal. The median, right, and caudate lobes were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. The left hepatic lobe was divided into sections for fixation in 10% buffered formalin, embedding in OCT, and storage for later use.

Collection of human liver tissue

Tissue was collected aseptically and specimens were stored under aseptic conditions at −80°C until use. Samples were from transplant recipients that had a diagnosis of nonalcoholic steatohepatitis (NASH) versus controls. Patients with a history of toxicant exposure (including ethanol), cardiac death donors, or other comorbidities were excluded from analysis. Anthropometric data for recipients are included in supplementary Table 1. Healthy livers were qualified by absence of macrosteatosis. Slides were read by an experienced liver pathologist blinded to experimental group and graded based on the method outlined by Kleiner et al. (21). All procedures were approved by the Medical University of South Carolina Institutional Review Board and conducted in accordance with University regulations.

Immunofluorescence microscopy

Upon the euthanization of an animal, sections of liver tissue from the left lateral lobe were placed in 10% neutral-buffered formalin for fixation, embedded in paraffin, and sliced. Sections were then washed in PBS twice for 5 min each before incubation in antibody. For SphK1, slides were incubated with 1:50 SphK1 antisera (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. After incubation, slides were washed with PBS three times for 10 min each before addition of secondary antibody (Alexa Fluor 488; Cell Signaling, Danvers, MA) for 1.5 h at room temperature and protected from light. After additional PBS washes, tissue samples were covered with glass coverslips and imaged using an Olympus FV10i scanning confocal microscopy.

For detection of p65, after heating as above, slides were submerged into xylene for three washes at 10 min each (with agitation halfway through each). Slides were then submerged into two 3 min rinses of 100, 95, and then 75% ethanol prior to being washed in PBS. Samples were blocked in 2% BSA for 1 h and incubated with 1:50 NFκB p65 (catalog #8242P, Cell Signaling) overnight at 4°C. After incubation, slides were washed with PBS three times for 10 min each before addition of secondary antibody (Alexa Fluor 594; Life Technologies, Carlsbad, CA) for 1.5 h at room temperature and protected from light. After additional PBS washes, tissue samples were covered with glass coverslips and imaged using an Olympus FV10i scanning confocal microscopy.

Oil Red O staining

For Oil Red O (ORO) staining of liver samples, the following protocol was used. Liver samples were embedded in OCT compound for frozen sectioning, cut into 6–10 micron sections, and placed on a charged slide. After all liver samples were cut, slides were washed quickly in distilled water to remove the OCT compound, and then washed quickly in 70% isopropl alcohol (five to ten dips). Next, slides were incubated in ORO solution (0.3 g ORO dissolved in 100 ml 36% triethyl phosphate solution) for 15 min. Excess ORO was washed off in 70% isopropl alcohol and slides were washed in distilled water. Last, slides were placed under a microscope to acquire images at 200x magnification. Images were quantified with Image-J software.

Measurement of cytokines in liver homogenates

Cytokines in mouse liver were determined using the Bio-Plex 2200 system (Bio-Rad, Hercules, CA). Assays were carried out
according to the manufacturer's instructions. Briefly, the liver sample was homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.5 mM EDTA, and a protease inhibitor cocktail. Homogenate was centrifuged, and supernatant was collected. The supernatant was then diluted 1:4 and incubated in a 96-well plate with magnetic antibody-coated beads for 30 min at room temperature while shaking. Plates were washed and incubated with detection antibodies for 30 min, followed by the addition of streptavidin-PE for 10 min. MCP1 and TNFα levels were measured using the Bio-Plex 2200 system. Each reaction was carried out in duplicate.

**Glucose and insulin measurements**

Mouse plasma glucose and insulin were measured in blood drops obtained from tail cuts as described (22).

**Pathological analysis of NASH**

Upon the euthanization of an animal, sections of liver tissue from the left lateral lobe were placed in 10% neutral-buffered formalin for fixation, embedded in paraffin, cut, placed on a glass slide, and heat-fixed. Hematoxylin and eosin staining was performed. Slides were read by an experienced liver pathologist blinded to experimental group and graded based on the semi-quantitative schema outlined by Kleiner et al. (21).

Neutrophils were identified by Leder stain (specific esterase or naphthol AS-D chloroacetate esterase). In brief, 4 μm-thick sections of formalin-fixed, paraffin-embedded liver were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated in a graded alcohol series. Specific staining was performed according to manufacturer's protocol and counterstained with Gill's hematoxylin. Positive cells were counted in 10 high-powered fields (HPFs) per section.

**F4/80 immunohistochemistry**

Formalin-fixed, paraffin-embedded sections were deparaffinized in Histoclear and rehydrated. Antigen retrieval was performed by incubation with proteinase K solution for 1 min at 37°C. After washing, endogenous peroxidases were blocked with Bloxall, followed by blocking with an endogenous streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Sections were then incubated with 1.5% normal rat serum, followed by incubation with 1:150 primary antibody (rat anti-F4/80, clone C57/1, Abcam, Cambridge, MA). Samples were then washed and incubated with biotinylated secondary antibody (Vector Laboratories) for an additional 30 min. Immunoperoxidase staining was performed with a diaminobenzidine substrate kit (Vector Laboratories), followed by washing and a counterstain with hematoxylin. Slides were then dehydrated through a graded alcohol series and xylen, and coverslipped with Cytoseal 60 mounting medium (Thermo Scientific, Pittsburgh, PA). Positive cells were counted as a ratio of total cells in 10 HPFs per section.

**Cell culture**

HepG2 cells were cultured under standard conditions, as suggested by ATCC. Isolation and culture of primary hepatocytes was performed according to standard protocols (23), and treatment with free fatty acids was described previously (22). For sphingosine-1-phosphate (S1P) treatment, S1P (catalog #BML-SL140-0001, Enzo Life Sciences Inc., Farmingdale, NY) was dissolved in 2 ml of methanol at 65°C by sonication, aliquoted, dried under a nitrogen stream, and stored at −20°C. For treatment, S1P was complexed to BSA (4 mg/ml) by incubation at 65°C for 30 min. This was then diluted as appropriate for S1P treatment.

**SphK1 activity assay**

SphK1 activity was measured in situ using the C17-sphingosine labeling method, as described (24).

**RNA interference**

All siRNAs, including human SphK1 (catalog #S10260455), S1P receptor 1 (S1PI) (catalog #S10391895), and S1P2 (catalog #S100061348) and negative control siRNA (catalog #1027416), were purchased from Qiagen. Their target sequences are shown as follows: 5′-CTG CGT CTG TAA GGC GCC TCT GTA CA-3′ for SphK1, 5′-CTC GGT CTC TGA CTA GGT CAA-3′ for S1PI, and 5′-TCC AGC ACT ATA ATT ATA-3′ for S1P2. HepG2 hepatocytes were transfected with these siRNAs, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The effects of RNAi on the expression of these genes were measured after 48–72 h of transfection and subsequent fatty acid treatment. In all cases, transfection conditions were optimized to achieve 70–80% reduction in the target mRNA (see supplementary Fig. 5). RNA interference experiments were performed on at least three independent occasions with comparable results.

**RNA analysis**

Total RNA was isolated from cells with Qiagen RNeasy mini kit (catalog #74106) or from mouse tissues with the use of Trizol (Invitrogen) according to manufacturer's instructions. The first-strand cDNA was synthesized from 1–2 μg of total RNA using the SuperScript™ first-strand synthesis system (Invitrogen) in a final volume of 20 μl of reverse transcription reaction. Real-time PCR was performed on an iCycler (Bio-Rad) as previously described (22). The reagents used in real-time PCR, including iQ™SYBR Green Supermix and gene-specific primers, were purchased from Bio-Rad and SABiosciences (Frederick, MD), respectively. Other noncommercial primers include: mouse Sphk1: 5′-ACA GTG GGC ACC TTC TTG G-3′, 5′-CTT CGT CAC CAG TGT AGA AGC CAA-3′; mouse MCP1: 5′-GGC TCA GCC AGA TGC AGT T-3′, 5′-GCT GGT GAT CCT CTT GT-3′; mouse collagen I: 5′-AGG AAA CCC GAG GTA TG-3′, 5′-TTG GGT GGC CCC TCG ACT CCT AC-3′; mouse S1PI: 5′-CTC CAC CGT GCT GCC CTA CA-3′, 5′-GGA GAT GTT CTT GCG GAA GTG CAG G-3′; mouse S1P2: 5′-GGC TGG TCA CCA TCT TCT CC-3′, 5′-CGT CTG CTG AGG ACC AGC AAC ATC-3′; human SphK1: 5′-GCA GAC CCA GCC TCC TTC ATG CAT-3′, 5′-GTA GCG AGA ACA GCA GGC TCA-3′; human TNFα: 5′-TGC GGC OCA GGC AGT CAG ATC AT-3′, 5′-CGG CCG TTC AGC CAG TGC AG-3′; human MCP1: 5′-TGC GTG CTT GCC GCT GCT CAT AG-3′, 5′-GGC CAT TGA TTG CAG CTG GC-3′; human TGB: 5′-GAA TAT TGA GGG CTT TCT CCC-3′, 5′-AGT GAA CCC GGT GAT GTC CA-3′; human collagen: 5′-ACA TGT TCA GGT TTG TCC ACC-3′, 5′-TGA TTG GGT GGA TGT CTT CCT GTG-3′; human S1PI: 5′-GAA GGG GGA GAA TAC GAA CA-3′, 5′-GCC AAA TGA ACC CTT TAG GAA-3′; human S1P2: 5′-CAC CTC GGA AGG ACA GAT AA-3′, 5′-CAT TGG AAG AGT CCG TCT CA-3′; and human actin: 5′-ATT GGC AAT GAG CCG TTC C-3′, 5′-GGT AGT TTG TGT GAT GGC ACA-3′. The cycle threshold (Ct) was determined with the supplied software, and standard curves were constructed for quantification. Quantification of a given gene, expressed as a relative mRNA level, was calculated after normalization to housekeeping genes (e.g., GAPDH RNA or 18s rRNA).

**Lipid measurements**

Medium from hepatocytes treated with palmitate or vehicle was analyzed for S1P content, as previously described (25).

**Immunoblot analyses**

Protein lysates from cultured cells or mouse tissues were analyzed by standard Western blot analysis protocol. The following

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primary antibodies were used: IκB (catalog #4814, Cell Signaling), p-IκB (catalog #2889, Cell Signaling), GAPDH (catalog #2118, Cell Signaling), actin (catalog #sc-1616; Santa Cruz Biotechnology), TNFα (catalog #sc-1350, Santa Cruz Biotechnology), SphK1 (catalog #29275; Cell Signaling). Band intensity was quantified using Scion Image software (http://www.brio.com/download/58-196-1.html).

**Statistical analysis**

All values here are expressed as mean ± SEM. For single pairwise comparisons of normally distributed data sets, a Student’s t-test was used. For multiple comparisons of means, a one-way ANOVA with Tukey-Kramer post hoc tests was used, and for ordinal variables a Chi-square test was used. All hypothesis testing was performed in GraphPad Prism 5 and P < 0.05 was chosen as a priori as indicative of statistical significance.

**Study approval**

Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and the Veteran’s Affairs Medical Center in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1996). Human liver samples were collected according to the Institutional Review Board guidelines of the Medical University of South Carolina. Informed consent from each human subject was obtained before sample collection.

**RESULTS**

Determinants of the transition from benign steatosis to steatohepatitis (NASH) remain unknown. A recent study from our laboratory demonstrated that dietary fat composition (i.e., saturated vs. unsaturated) had a profound effect on deleterious liver outcomes, including ER stress, liver weight, and hepatic steatosis, which are components of NAFLD (22, 26–30). Moreover, other studies in our laboratory and others indicated that palmitate (C16:0), but not oleate (C18:1), increased expression of SphK1 in skeletal muscle, and that the enzyme was required for expression of proinflammatory genes in primary skeletal muscle cells and also in skeletal muscle in mice on a high-fat diet (7, 13). Additionally, we demonstrated that high-fat diet-mediated induction of proinflammatory cytokines in skeletal muscle was impaired in SphK1−/− mice (13). Together, these studies provided rationale for testing our hypothesis that high saturated fat feeding may promote inflammation in NAFLD via induction of SphK1.

**SphK1 induction in vivo and role in experimental NASH in mice**

To test our hypothesis that a diet high in saturated fat may promote hepatic steatosis, we implemented a high saturated fat diet (HSFD) in adult C57BL/6j WT mice beginning at 8 weeks of age and ending at 24 weeks of age, for a total of 16 weeks of high saturated fat feeding. As a control, we implemented an isocaloric diet (CD) in which the calories from fat were replaced by low-glycemic starch. After 16 weeks of HSFD feeding, we observed an increase in liver weight (Fig. 1A) and a significant increase in macrosteatosis, as measured by ORO staining (Fig. 1B, C). During the course of these studies, mice lacking functional SphK1 (SphK1−/−) were also placed on this diet, and they demonstrated protection from these outcomes (Fig. 1A–C), including both no increase in liver weight and slight yet significant reduction in total staining. Moreover, the ORO distribution throughout the organ was less homogenous as compared to the WT mice. Under HSFD feeding conditions, both WT and SphK1−/− mice demonstrated weight gain and an increase in fasting glucose (supplementary Fig. 1; supplementary Table 2), though these increases were slightly blunted in SphK1−/− mice. Additionally, WT mice became hyperinsulinemic, with fasting insulin approximately 2.5-fold increased by the HSFD, but the SphK1-null mice showed less than a 50% increase (supplementary Fig. 1; supplementary Table 2). These data suggest that the HSFD promoted the general metabolic context for NAFLD in WT mice, and that SphK1 may play some role in this process, which is consistent with previous findings in SphK1−/− mice on obesogenic diets, namely, protection from insulin resistance (16). This study also demonstrated no protection from other parameters of metabolic dysfunction, including plasma free fatty acids, suggesting the roles of SphK1 in metabolic aberration are diverse in the obese context.

A potential role for SphK1 in NAFLD was supported by the observed elevation in SphK1 mRNA by approximately 2-fold in mouse liver homogenates from HSFD-fed mice versus CD-fed mice (Fig. 1D). To test potential relevance of this to human disease, we also analyzed SphK1 message levels in samples from healthy lean human subjects and humans with NASH. Human liver biopsies from individuals with context-appropriate anthropometrics (supplementary Table 1) were scored for NASH as described by Kleiner et al. (21). We observed similar fold induction of SphK1 in human NASH livers relative to lean controls (Fig. 1D); however, these studies were performed in liver homogenates, which did not provide information as to whether this induction occurred in hepatocytes or another cell type. Thus, we performed immunohistochemistry on liver sections from mice fed control versus HSFD diets to determine the cellular and subcellular localization of SphK1. These images showed that HSFD-fed mice exhibited increased SphK1 enzyme in hepatocytes, and moreover, the enzyme was localized in puncta at the plasma membrane (Fig. 1E, red arrows), which is consistent with an activated state of the enzyme (31, 32). We also noted high expression of the enzyme in non-hepatocyte cells, which we speculated were immune cells infiltrating into the liver parenchyma (Fig. 1E, blue arrows). Therefore, analysis of SphK1 protein or activity, or levels of its product, S1P, in mouse liver homogenates would be potentially confounded by high levels of non-hepatocyte expression.

Because S1P can activate proinflammatory signaling pathways and immune cell chemotaxis (33, 34), and, moreover, as the defining and clinically deleterious element of NAFLD is progression to NASH, which is defined by inflammation, we hypothesized that SphK1 might mediate disease progression. To test this, we assessed several proinflammatory outcomes associated with NASH in liver homogenates, including activation of NfkB signaling,
Sphingosine kinase 1 mediates hepatic inflammation in NAFLD. However, this was not observed in SphK1-null mice (Fig. 2A). Because the liver is comprised of several cell types, we also assessed hepatocyte-specific p65/NFκB. Staining for p65 in liver sections from WT mice on CD versus HSFD revealed that HSFD feeding induced elevation of p65 production of proinflammatory cytokines and/or chemotactic proteins, and immune cell infiltration. We observed NFκB activation by assessing phosphorylation of IκB in liver homogenates. WT mice fed the HSFD demonstrated increased phosphorylation of IκB relative to controls; however, this was not observed in SphK1-null mice (Fig. 2A).
Fig. 2. SphK1-null (or SphK1<sup>−/−</sup>) mice were protected from proinflammatory cytokine production response to high saturated fat feeding. A: Representative immunoblot of the phosphorylation of IκB in liver homogenate (n = 5–6). *P < 0.05 versus CD group; #P < 0.05 versus WT group. B: Quantification of immunoblot expressed as fold change over WT, CD. C: Tnfa mRNA abundance in liver homogenates as measured by real-time PCR. D: Tnfa protein abundance in liver homogenates as measured by Bioplex in the Materials and Methods. *P < 0.05 versus CD group; #P < 0.05 versus WT group. E: MCP1 mRNA abundance in liver homogenates as measured by quantitative PCR. F: MCP1 protein abundance in liver homogenates as measured by Bioplex in the Materials and Methods. For (A–D), n = 5–6; *P < 0.05 versus CD group; **P < 0.01 versus CD group; #P < 0.05 versus WT group; ##P < 0.01 versus WT group.

throughout the cell, and this was not observed in SphK1<sup>−/−</sup> mice (Fig. 2B). Because signaling through NFκB regulates proinflammatory cytokine expression, we hypothesized that these would be increased in WT mice on the HSFD and that this might also be attenuated in SphK1<sup>−/−</sup> mice. Quantification of message encoding TNFα revealed a 2-fold increase in message that was completely attenuated in SphK1-null mice, with a commensurate increase in TNFα protein (Fig. 2D, E). This was similar for MCP1 (Fig. 2E, F). No differences in cytokine expression were observed between the two strains on normal chow (supplementary Fig. 3). Combined, these data supported that the
high saturated fat feeding promoted proinflammatory signaling in mice in a manner dependent on SphK1, and suggest that bona fide inflammation may also lie downstream from these pathways.

To test whether the function of SphK1 in regulating cytokines and chemoattractants may manifest in immune cell recruitment to liver and/or pathological hepatocyte damage, we assessed neutrophils and macrophages in liver sections from WT or SphK1-null mice. While HSFD-fed WT mice showed significant neutrophil accumulation, as assessed by specific esterase staining, this was attenuated in SphK1-null mice (Fig. 3A, B). Quantification of F4/80 positive cells in 10 high-power microscope fields indicated a similar pattern (Fig. 3C). Thus, SphK1 appeared to underlie a subset of inflammatory processes in NASH. The poor functional outcomes of hepatic inflammation include decreased liver function. Serum alanine aminotransferase, a clinical measure of liver function, exhibited elevation in NASH that was attenuated in SphK1-null mice (Fig. 3D).

Roles of SphK1 and S1P in proinflammatory signaling in hepatocytes

Because the above findings were taken from liver homogenates, which contain material from several cell types, we sought to understand what portion of these events may occur specifically in hepatocytes. Previous studies in our lab showed that HSFD feeding altered plasma free fatty acid composition in mice, increasing the proportion of saturated fatty acids relative to unsaturated (22), which has also been reported in humans (35). To test whether the HSFD could be translated to a cell culture system for more mechanistic studies, isolated mouse hepatocytes were treated with palmitate, the most abundant saturated fatty acid in mouse and human plasma, and SphK1 message was assessed. Consistent with our observations in vivo, palmitate elevated SphK1 message by 2-fold (Fig. 4A). SphK1 protein [top band, ~50 kDa (36)] was elevated by 12 h and more robustly at 24 h after palmitate treatment relative to BSA-treated cells (Fig. 4B). To test the effect of

![Fig. 3. SphK1-null mice were protected from immune cell infiltration response to high saturated fat feeding. A: Specific esterase staining of neutrophil infiltrate in mouse liver sections. B: Quantitative analysis of neutrophils in liver sections derived from 10 HPFs. C: Quantification of F4/80 positive cells in liver sections from 10 HPFs. D: Serum alanine aminotransferase levels. *P < 0.05, WT HSFD versus WT CD; #P < 0.05, SphK1-null HSFD versus WT HSFD, respectively. Data are presented as mean ± SEM; n = 5–6.](image-url)
Fig. 4. Palmitate induced SphK1 mRNA and increased SphK1 products, while S1P induced proinflammatory signaling in primary mouse hepatocytes, both WT and SphK1−/−. A: Palmitate (Pal) increased SphK1 message in hepatocytes. SphK1 message abundance is shown as fold change over cells treated with BSA. *P < 0.05 versus BSA; n = 5. B: SphK1 protein was increased in hepatocytes treated with palmitate relative to BSA-treated controls. C: Dihydrosphingosine-1-phosphate was increased in cell pellets treated with palmitate relative to BSA-treated cells (approximately 500,000 cells), n = 3, while S1P increased in the media (picomoles per milliliter), n = 3. D: Representative immunoblots of phosphorylated IkBα versus IkBα indicating NFκB activation in response to S1P or vehicle (Veh) control. E: Quantification of immunoblots of phosphorylated IkBα versus IkBα as shown in (C). **P < 0.01 versus Veh, n = 5. Tnfα mRNA (F) and Mcp1 mRNA (G) were induced by S1P in primary hepatocytes. Message RNA abundance is shown as fold change over Veh group. *P < 0.05 versus Veh; **P < 0.01 versus Veh; n = 5. Data are presented as mean ± SEM. Tnfα mRNA (H) and Mcp1 mRNA (I) were induced by S1P in SphK1−/− primary hepatocytes. Message RNA abundance is shown as fold change over Veh group. *P < 0.05; n = 3. Data are presented as mean ± SEM.
this elevation on sphingolipid profiles, we measured the products of SphK1, dihydro sphingosine-1-phosphate and S1P, in cells and media of palmitate-treated cells. S1P was not detectable in cell pellets under any condition; however, dihydro sphingosine-1-phosphate was increased approximately 6-fold in cell pellets treated with palmitate relative to BSA-treated cells. In contrast, media showed an increase in both species induced by palmitate treatment, from undetectable to 0.8 or 1.2 pmol/ml for dihydro sphingosine-1-phosphate and S1P, respectively (Fig. 4C).

These data indicated that palmitate not only increased SphK1, but also increased extracellular S1P. Therefore, to test whether extracellular S1P might induce proinflammatory signaling in primary hepatocytes, cells were treated with S1P as a BSA complex. In keeping with results in vivo, S1P treatment led to IkB phosphorylation (Fig. 4D, E) and increased TNFα message (Fig. 4F), which was dose-responsive and occurred as a bell-shaped curve, in keeping with previous observations (37) (supplementary Fig. 4). Additionally, S1P treatment induced mRNA for MCP1 (Fig. 4G). There were no differences in basal expression of TNFα between WT and SphK1−/− primary hepatocytes, and SphK1−/− primary hepatocytes also showed increased TNFα in response to S1P (Fig. 4H). Moreover, while we noted a 2-fold basal elevation of MCP1 in the SphK1−/− cells (not shown), they responded robustly to palmitate treatment by inducing MCP1 message to the same degree as WT hepatocytes. This suggests that the S1P signaling machinery remains intact in SphK1-null cells, further supporting that the failed upregulation of proinflammatory cytokines derives from the inability of palmitate to induce SphK1. Induction of signaling through NfkB and concomitant upregulation of cytokine message suggest that S1P may, at least in part, initiate proinflammatory signaling. Treatment with palmitate did not induce TNFα or MCP1 mRNA. Similarly, neither palmitate nor S1P treatment increased protein for these cytokines in these time frames (12–16 h). This may be due to the observed loss of viability of these cells with high concentrations of palmitate. Therefore, to investigate the requirement for SphK1 for palmitate-induced cytokine production mechanistically, we used HepG2 cells.

**Receptor-mediated S1P proinflammatory signaling in HepG2 cells**

Together with the in vivo data, the data in hepatocytes supported a model whereby saturated fatty acids increase hepatocyte expression of SphK1 and production of S1P. Moreover, as extracellular S1P also induced proinflammatory cytokine mRNA, this suggests a receptor-mediated mechanism. To test this, we used HepG2 cells, which are both more amenable to transfection than primary hepatocytes and able to maintain viability even under conditions of long treatments (e.g., 16 h) with palmitate. First, to test whether the HepG2 system would recapitulate the above findings, we treated cells with palmitate. Similar to hepatocytes, palmitate induced sphingosine kinase (Fig. 5A). Additionally, to test SphK1 activity in cells, cells were treated with palmitate for 16 h, and then acutely labeled with a 17 carbon-containing sphingosine (C17-sphingosine) and assessed for levels of C17-S1P in media. By 20 min, media from palmitate-treated cells contained roughly double the product compared with BSA-treated cells (2.43 ± 0.65 pmol/ml vs. 1.21 ± 0.05 pmol/ml, respectively). Direct treatment with S1P increased NfkB activation and TNFα mRNA abundance (Fig. 5B, C). We therefore used this system to assess whether actions of palmitate required SphK1. Cells were treated with palmitate with and without siRNA-mediated knockdown of SphK1. Knockdown efficiency was greater than 80% (supplementary Fig. 5), and completely attenuated palmitate-induced NfkB activation and TNFα abundance (Fig. 5E, F). This is strongly supportive of a linear relationship between palmitate, SphK1, S1P, and proinflammatory signaling. In this system, we observed no induction by either palmitate or S1P of MCP1 message. Notwithstanding this, the system recapitulated the most important findings from in vivo and primary cells, namely the saturated fat-dependent upregulation of SphK1 that was required for proinflammatory signaling through production of S1P.

TNFα signaling and NfkB activation have been ascribed to SphK1/S1P in other contexts; however, data support that this may be either dependent on or independent of S1P receptors and may depend on tissue and/or disease context (38, 39). To test whether S1P-dependent proinflammatory signaling occurred through S1P receptors in our system, we targeted S1P receptors for siRNA knockdown and treated cells with palmitate. Transfection of HepG2 cells with siRNA decreased message of S1P1 and S1P2 to 20 and 30% of control values, respectively (supplementary Fig. 6). Knockdown of S1P1, but not S1P2, prevented palmitate-mediated phosphorylation of IkB (Fig. 6A). We also tested whether S1P1 may mediate increase in TNFα message. Knockdown of S1P1, but not S1P2, prevented palmitate-induced increase in TNFα message (Fig. 6B, C), leading to the conclusion that S1P-dependent proinflammatory signaling occurs through S1P1.

**DISCUSSION**

In this study, we demonstrated that high saturated fat feeding in mice recapitulated defining features of NASH, presenting a novel and potentially broadly useful model of this disease. Furthermore, in both human and mouse NASH, elevation of SphK1 message was observed. These studies showed that mice lacking functional SphK1 were protected from major characteristics of NASH. Further mechanistic studies in isolated hepatocytes and/or HepG2 cells supported that SphK1/S1P-dependent inflammation, as measured by levels of TNFα and MCP1, may occur, at least in part, through NfkB signaling downstream from S1P1. More in-depth mechanistic approaches will be required to fully understand the mechanism of SphK1/S1P-mediated activation of NfkB signaling, including studies in mice with hepatocyte-specific deletion of SphK1, to dissect the precise roles of SphK1 in NAFLD in vivo; however, because S1P1 currently serves as a therapeutic target for
other inflammatory disease processes, these studies may provide rationale for further investigation into potential strategies targeting the SPHK1/S1P pathway for NAFLD treatment. It should be stated, however, that findings herein may be limited to situations where individuals consume a diet high in saturated fat. In fact, these data may shed light on well-known associations between saturated fat and metabolic disease severity and, in particular, NAFLD (28).

Some limitations of this study include the use of a whole-body knockout, in which the metabolic characteristics of the HSFD, including weight gain, percent increase in fasting glucose (though total fasting glucose was higher than in the WT mice), and hyperinsulinemia, were partially attenuated. Several studies support roles for SphK1 in regulating diabetes-associated disease processes in other tissues. Some functions appear protective, for example, ameliorating muscle insulin resistance (40), promoting pancreatic β-cell survival (8), and blocking TGFβ-dependent fibrosis in kidney (41). On the other hand, a few reports also reveal deleterious roles for this pathway, such as mediating glucose-induced inflammation in endothelial cells and IL-6 production and signaling in skeletal muscle and adipose tissue, respectively (13, 33). Therefore, whether the net contribution of SphK1 is ultimately protective or deleterious in the obese/diabetic context remains unresolved, and identification of individual receptors that mediate distinct effects may facilitate targeting this pathway for specific clinical interventions.

The hepatocyte-specific SphK1−/− animal is being generated in our laboratory, and this will allow the parsing of hepatocyte-specific contributions to NAFLD. However, though steatosis was partly attenuated, it was still significantly increased relative to controls, and thus we suspect the contributions of S1P from other tissues may contribute, but modestly, to the liver pathology observed in the HSFD model system. Along these lines, because of increased expression of SphK1 in non-hepatocyte cells (Fig. 1), measurements of SphK1 activity and/or S1P levels in liver homogenates would not be conclusive as to the contribution of hepatocytes. We also have studies currently...
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We did not observe consistent elevation of MCP1 in HepG2 cells. However, despite these inconsistencies in details between the models, the major conclusion is that saturated fat, at least in part through SphK1 induction and/or S1P production, increases proinflammatory signaling. Further study will be required to distinguish between these and/or other mechanisms underlying the differences in cytokine protein expression between the in vivo and cell culture systems; however, the profound protection attained in the knockout mouse suggests that whichever cell types are involved, this pathway could serve as a valuable clinical target for anti-inflammatory pharmacological agents.

Though further study will be required to determine the mechanisms by which SphK1 regulates inflammation in vivo in NAFLD, a regulatory role for the SphK1/S1P axis in regulating immune cell infiltration in liver is consistent with a previous study that prevention of S1P catabolism in a S1P lyase-deficient mouse, which elevated S1P, had elevation of total neutrophils but impaired migration, as neutrophils accumulated within the sinusoids, but did not infiltrate the parenchyma (34). We observed a parallel phenomenon in Sph1K-null mice subjected to high-fat feeding, where neutrophils failed to emigrate from the hepatic sinusoids. We thus propose that a chemotactic S1P gradient may be required for successful emigration of neutrophils out of hepatic sinusoids, which might be disrupted in both an SPHK1-null mouse (deficient in S1P) and an S1P lyase-deficient mouse (containing a universal elevation of S1P); however, this remains to be tested.

Another limitation is that, while MCP1 was elevated in vivo in HSFD feeding (mRNA and protein) and in primary hepatocytes (mRNA only), we did not observe consistent elevation of MCP1 in HepG2 cells. However, despite these inconsistencies in details between the models, the major conclusion is that saturated fat, at least in part through SphK1 induction and/or S1P production, increases proinflammatory signaling. Further study will be required to distinguish between these and/or other mechanisms underlying the differences in cytokine protein expression between the in vivo and cell culture systems; however, the profound protection attained in the knockout mouse suggests that whichever cell types are involved, this pathway could serve as a valuable clinical target for anti-inflammatory pharmacological agents.

Because of these limitations to mechanistic understanding in the animal model, we transitioned to primary mouse hepatocytes for some studies. These cells are not amenable to transfection, and, when treated with palmitate, they began to lose viability at later time points. Therefore, we also employed HepG2 cells. Thus, three models of NASH were used, and there were some inconsistencies between them. First, in contrast to the mouse model, we did not observe protein increases in cytokines in the cell models. This may arise from different kinetics of the in vivo versus cell culture systems, i.e., in vivo, hepatocytes were exposed to moderately elevated saturated fat over 16 weeks, whereas hepatocytes in culture did not robustly survive palmitate treatment after 24 h, and HepG2 cells began to detach by 24 h. Another possibility is that in vivo, the cytokines derive from the immune cells present, which are rich in proinflammatory cytokines. Studies in mice bearing cell type-specific SphK1 deletion will distinguish between these two possibilities. However, it seems unlikely that message of these cytokines would increase if no protein were to be produced. A third possibility is that protein expression may require several mechanisms, including cross-talk among hepatic cell types in vivo and/or a parallel pathway by which palmitate enables translation of the mRNAs.

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A particularly interesting aspect of this work is the disease model system used. Suitable mouse models for NASH

![Figure 6](image_url) Palmitate (Pal) induced proinflammatory signaling in HepG2s in an S1P1-dependent manner. A: Immunoblot representing siRNA-mediated knockdown of S1P1, but not S1P2, blocked increased p-IκBα and increased TNFα message (B, C). **P < 0.01 versus the control siRNA (Ctrl si) and BSA group; #P < 0.05 versus the Ctrl si and Pal group, respectively. Data are presented as mean ± SEM, n = 5.
have been difficult to develop, and we serendipitously discovered steatotic livers in mice being fed this diet for another experimental purpose. Further investigation revealed that this milk-fat-based HSFD, but not a typical lard-based obesogenic diet (both are 60% kcal from fat) increased hepatic expression of prosteatotic genes, including SCD1, CD36, and PPARγ (30); moreover, as presented here, proinflammatory signaling and immune cell infiltration also occur in this model. As these are key components of clinical NAFLD that cause concern (rather than steatosis alone, which may remain benign in many cases), we propose that this model may well serve further study of NASH using mice. This is also supported by the observation that these mice, with elevations in weight, fasting glucose, and fasting insulin, have the appropriate metabolic context for NAFLD in humans. A major health concern in NASH is progression to fibrosis. Mice were maintained on these diets for 16 weeks in this study. Liver homogenates demonstrated SphK1-dependent upregulation of TGFβ (not shown), though we observed little, if any, bona fide increase in these mouse livers. However, we speculate that longer time points may also lead to fibrotic livers, and this is currently being addressed in our laboratory. This model also caused ER stress (22), which is an increasingly appreciated aspect of NASH in humans (28).

In summary, this work demonstrates that an obesogenic diabeticogenic diet high in saturated fat promotes NASH, at least in part, through SphK1 and that this enzyme, which is induced in NASH in vivo in mice and humans and also by saturated fatty acids in hepatocytes, mediated proinflammatory signaling through SIP1. As this pathway currently serves as a therapeutic target for disease in humans, further mechanistic studies of SphK1-mediated inflammation in NASH may reveal novel routes of NAFLD treatment.

The SphK1 mice were originally given by Dr. Richard Proia, National Institute of Diabetes and Digestive and Kidney Diseases, and maintained by the animal core of the Center of Biomedical Research Excellence in Lipidomics and Pathobiology at the Medical University of South Carolina. Lipid measurements were performed at the lipidomics core facility of the COBRE in Lipidomics and Pathobiology.

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