Sphingosine kinase 1 regulates adipose proinflammatory responses and insulin resistance

Jing Wang, Leyla Badeanlou, Jacek Bielawski, Theodore P. Ciaraldi, and Fahumiya Samad

Department of Cell Biology, Torrey Pines Institute for Molecular Studies, San Diego, California; Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina; Veterans Affairs San Diego HealthCare System, La Jolla, California; and Department of Medicine, University of California San Diego, La Jolla, California

Submitted 4 October 2013; accepted in final form 24 January 2014

Wang J, Badeanlou L, Bielawski J, Ciaraldi TP, Samad F. Sphingosine kinase 1 regulates adipose proinflammatory responses and insulin resistance. Am J Physiol Endocrinol Metab 306: E756–E768, 2014. First published January 28, 2014; doi:10.1152/ajpendo.00549.2013.—Adipose dysfunction resulting from chronic inflammation and impaired adipogenesis has increasingly been recognized as a major contributor to obesity-mediated insulin resistance, but the molecular mechanisms that maintain healthy adipocytes and limit adipose inflammation remain unclear. Here, we used genetic and pharmacological approaches to delineate a novel role for sphingosine kinase 1 (SK1) in metabolic disorders associated with obesity. SK1 phosphorylates sphingosine to form sphingosine 1 phosphate (S1P), a bioactive sphingolipid with numerous roles in inflammation. SK1 mRNA expression was increased in adipose tissue of diet-induced obese (DIO) mice and obese type 2 diabetic humans. SK1 deficiency increased markers of adipogenesis and adipose gene expression of the anti-inflammatory molecules TNF-α and IL-10 and adiponectin and reduced adipose tissue macrophage (ATM) recruitment and proinflammatory molecules TNFs and IL-6. These changes were associated with enhanced insulin signaling in adipose and muscle and improved systemic insulin sensitivity and glucose tolerance in SK1−/− mice. Specific pharmacological inhibition of SK1 in WT DIO mice also reduced adipocyte and ATM inflammation and improved overall glucose homeostasis. These data suggest that the SK1-S1P axis could be an attractive target for the development of treatments to ameliorate adipose inflammation and insulin resistance associated with obesity and type 2 diabetes.

Sphingosine kinase 1; sphingosine 1 phosphate; adipose inflammation; insulin resistance; adipogenesis

The adipose tissue lies at the heart of the metabolic syndrome, and growing evidence indicates that obesity-associated metabolic disease occurs because of dysregulation of metabolic, endocrine, and immune functions of the adipose tissue (14, 19, 28). Impaired adipogenesis due to nutritional overload causes lipid deposition in ectopic tissues, including liver, muscle, and pancreases, and disrupts normal metabolic function (14, 20, 45, 47). Adipose inflammation driven by both adipocytes and infiltration and activation of adipose tissue macrophages (ATM) further disrupts normal adipose function and is linked to the development of insulin resistance/type 2 diabetes (T2D) (28, 29). Understanding the mechanisms that maintain metabolically healthy adipocytes and limit chronic adipose inflammation may identify novel targets for the treatment of obesity-related metabolic complications.

The bioactive sphingolipid sphingosine 1 phosphate (S1P) regulates signaling pathways crucial to cell growth, survival, migration, immune cell trafficking, angiogenesis, and inflammation (25, 41, 42). S1P is generated by phosphorylation of the ceramide metabolite sphingosine by two sphingosine kinases (SK1, SK2). S1P is synthesized by most cells, including platelets, erythrocytes, endothelial cells, mast cells, and macrophages (25, 41, 42). The major cellular and secreted source of S1P is derived from SK1 and mediates its functions through five cell-surface GPCRs (S1P1–5) or poorly defined intracellular targets (25, 41, 42). Sphingolipid metabolism generating ceramide and S1P can be activated by proinflammatory cytokines, growth factors, and free fatty acids (FFAs) present in the adipose tissue milieu in obesity (28, 29). In obese mice, ceramide levels are increased in plasma (36, 38) and adipose tissue (38, 43). Ceramide and S1P levels are also increased in the adipose tissue and plasma of obese humans (6, 7, 16, 21, 37). In lipid-infused or diet-induced obese (DIO) animal models, inhibition of de novo ceramide synthesis reduces protein phosphatase 2A activity, activates insulin-mediated Akt phosphorylation, and leads to increased insulin sensitivity (17, 37, 49). SK1/S1P signaling, with crucial roles in inflammation and immune cell trafficking (25, 41, 42), has the potential to contribute to insulin resistance via mechanisms that overlap with or are distinct from ceramide. However, its role in obesity-mediated adipose inflammation and insulin resistance has not been clearly defined.

The contribution of specific sphingolipids to insulin resistance is unclear because the synthesis of bioactive sphingolipids is regulated by a network of interconnected pathways, and an alteration in levels of one sphingolipid affects others. Studies have suggested that increased SK1 activity and S1P negatively regulate ceramide levels (22, 26) and that inhibition of SK1 increases ceramide (30). However, this is not always the case in vivo, where the network of regulatory pathways is more complex. HFD-fed SK1-transgenic mice showed no significant changes for muscle S1P levels, whereas ceramide levels were decreased (8). Mice deficient for S1P lyase, the enzyme that breaks down S1P, had increased levels of both S1P and ceramide (4). Improved insulin resistance after inhibition of de novo ceramide synthesis in genetically obese ob/ob and DIO mice resulted in decreased plasma ceramide and a modest but significant decrease in S1P (49). These studies underscore the complexity of the ceramide-S1P rheostat in vivo and the challenge in elucidating in vivo roles for specific sphingolipids in the metabolic complications of obesity.
con or nondiabetic by their response to a 75-g oral glucose tolerance test. Subjects were recruited from diabetes clinics and classified as diabetic or nondiabetic by type 2 diabetes (T2D); n = 6 –10 ± SD. **P < 0.01 for LFD vs. HFD. BD, below detection threshold. G: SK1 mRNA in adipose tissues of obese nondiabetics (ND) compared with type 2 diabetes (T2D); n = 6 –10 ± SD. ***P < 0.001 for LFD vs. HFD. BD, below detection threshold. G: SK1 mRNA in adipose tissues of obese nondiabetics (ND) compared with type 2 diabetes (T2D); n = 6 –10 ± SD. **P < 0.01 ND vs. T2D. Clinical characteristics of research subjects are given in Table 1.
Sphingolipid analysis. Ceramide, sphingosine, and S1P were analyzed by HPLC-tandem mass spectroscopy, as described (5, 49), at the Lipidomics core facility, Medical University of South Carolina. Briefly, plasma or tissue homogenates (in 50 mM Tris, pH 7.4, 25 mM KCl, 0.25 M sucrose, and 0.5 mM EDTA) were spiked with internal standards and extracted into a one-phase neutral organic solvent system (ethyl acetate-isopropanol-water, 60:30:10, vol/vol). The solvents were evaporated, and the sample was reconstituted in methanol and analyzed with an HP1100/TSQ 7000 LC/MS system. Quantitative analysis of analytes was performed in positive multiple reaction monitoring mode based on calibration curves generated by spiking an artificial matrix with known amounts of target analytes, synthetic standards, and internal standards. Sphingolipid levels were normalized to total protein levels (1 mg protein/sample).

Adipose tissue fractionation and adipogenesis. EAT was washed and minced in PBS containing 0.5% BSA and incubated at 37°C with

Fig. 2. SK1 regulates adiposity and steatosis. A: body and organ weights (B and C) of male wild-type (WT) and SK1−/− mice fed a HFD for 16 wk. For A–C, n = 10 ± SD. D–G: hematoxylin and eosin-stained sections of liver (D: WT; E: SK1−/−) and EAT (F: WT; G: SK1−/−) of HFD fed mice. H and I: adipocyte diameter distribution of adipocyte size in EAT of HFD-fed WT and SK1−/− mice. J: gene expression of adipogenic markers in adipocyte cultures from adipose tissue SVF 12 days postdifferentiation. K: Western blot of adipose PPARγ phosphorylation on Ser112 (top) and non-phospho-PPARγ (bottom); n = 6 ± SD. The bands of WT and SK1−/− in the Western blot are from the same blot and at the same exposure level. The separating white space denotes membrane splicing for clear presentation. *P < 0.05, **P < 0.01 WT vs. SK1−/−.
collagenase (1 mg/ml in PBS/0.5% BSA) for 20 min on a shaking platform. The mixture was filtered through a nylon filter (pore size 250 μm) and centrifuged (5 min, 200 g at 4°C), and floating cells and pellets were recovered as mature adipocyte and the stromal vascular fraction (SVF), respectively. The adipocyte fraction was further washed twice with PBS prior to mRNA isolation. The SVF was incubated in RBC lysis buffer (5 min), resuspended in DMEM/h 10% FBS, and cultured for three passages before differentiation. Adipogenesis was induced 2 days after preadipocyte culture was 100% confluent by treating cells with 200 nM insulin, 250 nM dexamethasone, and 0.5 nM isobutylmethylxanthine (Sigma-Aldrich) for 3 days and then for an additional 3 days in the absence of differentiating agents, with media replaced every 2 days. Twelve days postdifferentiation, cells were harvested for mRNA isolation and gene analysis.

Flow cytometry and CD11c+ selection. Flow cytometry of EAT-derived SVF cells was performed as described previously (2). Cells were resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS containing 1% FCS and 1 mM EDTA) and stained at 4°C for 30 min with fluorophore-labeled monoclonal antibodies to CD11b and CD11c (eBioscience) in the presence of Fc receptor-blocking antibodies (anti-CD16/CD32; eBioscience). Propidium iodide (Invitrogen) was added for live cell gating. Cells were washed, fixed in 1% formaldehyde, and analyzed on an LSR-II cytometer (BD Biosciences) with data processing using FlowJo (Tree Star). CD11c+ cells were positively selected from EAT SVF cells using anti-CD11c paramagnetic microbeads (Miltenyi Biotech).

Real-time quantitative RT-PCR. cDNAs synthesized from total RNA (Ultraspec RNA isolation system; Biotecx Laboratories) were analyzed with gene-specific primer sets (Invitrogen) and SYBR Green PCR Master mix (PerkinElmer) in an iCycler (Bio-Rad) (2, 49). Relative gene expression levels were calculated after normalization to β-actin using the ΔΔCT method (Bio-Rad). The expression of the housekeeping gene β-actin was stable in the samples measured. Separate control experiments demonstrated that the efficiencies of target and reference (i.e., β-actin) amplifications were equal, thus validating the use of ΔΔCT for all of the transcripts that were measured.

Histology and immunohistology. Formalin-fixed, paraffin-embedded sections (6 μm) of adipose and liver were stained with hematoxylin and eosin. Average adipocyte diameters were calculated by measuring the diameters from six random microscopic fields from each mouse using a image analysis software (SPOT; Diagnostic Instruments). For immunohistology, formalin-fixed paraffin sections of adipose tissues were incubated overnight at 4°C with the primary antibody rat anti-mouse F4/80 (Serotec). Negative controls were done without the primary antibody. The slides were then washed and treated sequentially with biotinylated goat anti-rat IgG (Jackson Immunoresearch), streptavidin-peroxidase conjugate (Zymed), and diaminobenzidine chromogen containing 0.03% hydrogen peroxide (Vector Laboratories). After rinsing in distilled water, the slides were counterstained with Gill modified hematoxylin for 20 s, rinsed, and mounted in GVA-mount (Zymed). Crown-like structures with positive macrophage staining were counted from six random microscopic fields from each mouse.

Statistical analysis. The statistical significance of differences between groups was analyzed using the unpaired Student t-test.

RESULTS

Adipose SK1/S1P expression increases with obesity and T2D. Because plasma FFAs are elevated in obesity, we first examined the effect of palmitate on primary adipocytes from C57BL/6 (WT) mice. Adipocytes cultured for 24 h with 1 mM palmitate showed increased levels of SK1 mRNA levels compared with control cells (Fig. 1A). These findings were confirmed in a DIO model where male C57BL/6J WT mice were fed a palmitate-rich HFD for 16 wk. EAT and adipocytes isolated from DIO mice showed increased SK1 mRNA levels compared with LFD-fed mice (Fig. 1, B and C). Consistent with SK1 expression, S1P levels were increased in EAT, isolated adipocytes, and plasma of DIO mice (Fig. 1, D–F).

To gain insight into the clinical relevance of these observations, we analyzed SK1 expression in adipose tissues of obese (BMI >30 kg/m²) humans with or without T2D (Table 1). SK1 mRNA levels in adipose tissue from obese T2D patients were significantly higher than in nondiabetic subjects (Fig. 1G), suggesting that the adipose SK1/S1P axis may be functionally significant in insulin resistance related to obesity and T2D.

SK1 regulates adiposity and causes insulin-resistance. To directly assess the contribution of SK1 to obesity and its metabolic consequences, we compared the physiological and biochemical consequences of a 16-wk HFD on WT and SK1−/− mice. WT and SK1−/− mice on the HFD showed no differences in food intake (data not shown) and gained weight at a similar rate (Fig. 2A). However, the SK1−/− mice had higher EAT and lower liver weights (Fig. 2, B and C) and decreased steatosis (Fig. 2, D and E). Histological examination
of EAT and quantification of adipocyte diameters revealed that DIO SK1<sup>−/−</sup> mice contained a larger number of smaller, less hypertrophic adipocytes than DIO WT mice (Fig. 2, F–I). Lipidomics analysis confirmed that S1P levels were decreased in the plasma, EAT, and adipocytes of DIO SK1<sup>−/−</sup> mice compared with WT mice (Fig. 3, A–C). In adipose tissues, no significant differences were observed between WT and SK1<sup>−/−</sup> mice in the transcriptional activity of enzymes that contribute to ceramide production via de novo ceramide synthesis [serine palmitoyl transferase (SPT); Fig. 4, A and B] or via hydrolysis of membrane sphingomyelin (acid sphingomyelinase and neutral sphingomyelinase; Fig. 4, C and D). Total ceramide and sphingosine levels in plasma, adipose tissues, and adipocytes were also similar in HFD-fed WT and SK1<sup>−/−</sup> mice (Fig. 4, E–G). Our data are consistent with those of Baker et al. (3), who showed that plasma ceramide levels were similar in WT and SK1<sup>−/−</sup> mice transgenic for human TNFα. Other studies suggest that C16 and C18 ceramides may play significant roles in the pathogenesis of obesity and related metabolic complications (35). The fatty acid composition of ceramide is regulated by a family of genes known as ceramide synthases (CerS), with six members (CerS1–6) identified thus far. Measurement of gene expression of CerS1–6 in adipose tissues indicates that, with the exception of CerS1, which was higher in SK1<sup>−/−</sup> mice, no significant differences were observed in any of the other CerS between WT and SK1<sup>−/−</sup> mice (Fig. 5, A–F). CerS1 preferentially drives C18 and C20 ceramide; however, by lipidomics analysis we found no differences in C18 or any of the other ceramide species measured in the plasma, EAT, or adipocytes of HFD-fed WT and SK1<sup>−/−</sup> mice (Fig. 5, G–I). Thus, SK1 deficiency specifically reduces plasma and adipose levels of S1P in this model of DIO mouse.

One mechanism that could contribute to adipocyte hyperplasia in SK1<sup>−/−</sup> mice is enhanced adipogenesis. To test this, we examined cells derived from the SVF of adipose tissue, which can differentiate into adipocytes in primary culture (1). SVF cultures derived from SK1<sup>−/−</sup> mice showed enhanced expression of adipogenic markers PPARγ, AP2, and GLUT4 and a parallel decrease in the preadipocyte marker Pref1 compared with cultures from WT mice (Fig. 2 J). Since a major

---

**Fig. 4.** A–D: serine palmitoyl transferase (SPT) and long-chain base subunit 2 (SPTLC2) and 3 (SPTLC3) (A and B) and acid sphingomyelinase (ASMase; C) and neutral sphingomyelinase (NSMase; D) mRNA levels in EAT of 16-wk HFD fed WT and SK1<sup>−/−</sup> mice. Ceramide and sphingosine levels in plasma (E), EAT (F), and adipocytes from epididymal adipose (G) of HFD-fed WT and SK1<sup>−/−</sup> mice; n = 5 ± SD.
mechanism that regulates adipogenesis is the phosphorylation status of PPARγ (11), we determined whether SK1 regulates PPARγ phosphorylation. Phosphorylation of PPARγ on Ser112 inhibits ligand binding by PPARγ and reduces its ability to promote adipogenesis (46). PPARγ Ser112 phosphorylation was reduced in adipose tissue from SK1−/− mice compared with WT mice (Fig. 2K), identifying a potential mechanism whereby SK1/S1P signaling might impair adipogenesis. These

Fig. 5. A–F: ceramide synthase 1–6 (CerS) gene expression in EAT of 16-wk HFD-fed WT and SK1−/− mice. Levels of individual ceramide species in plasma (G), epididymal adipose (H), and adipocytes (I) isolated from EAT of HFD-fed WT and SK1−/− mice; n = 5 ± SD.
Fig. 6. SK1 contributes to HFD-mediated glucose tolerance and insulin resistance. Fasting plasma insulin (A), glucose (B), free fatty acids (FFA; C), and triglycerides (TG; D) in male WT and SK1<sup>−/−</sup> mice fed a HFD for 16 wk. Glucose tolerance test (GTT; E), plasma insulin after GTT (F), and insulin tolerance test (ITT; G) in 16-wk HFD-fed WT and SK1<sup>−/−</sup> mice. For GTT and ITT, mice were injected ip with either insulin (0.75 U/kg body wt Himulin) or glucose (2 g/kg body wt), and blood glucose was determined at indicated times. For A–G, n = 10 ± SD. Phosphorylated Akt in EAT (H), muscle (I), and liver (J) of HFD-fed WT and SK1<sup>−/−</sup> mice. For analysis of Akt, mice were fasted for 6 h, injected with 0.75 U/kg insulin via the tail vein, and euthanized 10 min later. Tissues were collected for Western blotting using antibodies to phosphorylated Ser<sup>473</sup> of Akt and Akt. For H–J, n = 5 ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, WT vs. SK1<sup>−/−</sup>. 

**E762 SPHINGOSINE KINASE 1 IN ADIPOSE INFLAMMATION**
observations suggest that in response to a HFD, deficiency of SK1 allows healthy expansion of the adipose tissue via recruitment of adipose precursor cells into the adipogenic program. An inability to store lipids in the adipose tissue during nutritional excess causes lipid accumulation and disruption of insulin signaling in extra adipose tissues (44, 45, 47). Because HFD-fed SK1/−/− mice displayed increased adipogenesis and adipose tissue expansion and decreased steatosis we asked whether glucose homeostasis was improved. Compared with HFD-fed WT mice, HFD-fed SK1/−/− mice had lower fasting plasma insulin and glucose (Fig. 6, A and B), but plasma FFA and triglyceride levels were similar in both genotypes (Fig. 6, C and D). In GTT, SK1/−/− mice more efficiently cleared an intraperitoneal bolus injection of glucose, demonstrating improved glucose tolerance (Fig. 6E), and plasma insulin levels at 15 and 30 min during GTT were also lower in SK1/−/− mice (Fig. 6F). SK1/−/− mice also demonstrated improved insulin sensitivity, shown by more efficient insulin-mediated suppression of plasma glucose in ITT (Fig. 6G). To determine the primary insulin-sensitive tissues that contributed to enhanced systemic insulin sensitivity, we analyzed insulin-mediated phosphorylation of Akt. Akt phosphorylation was higher in the EAT, muscle, and liver of DIO SK1/−/− than DIO WT mice following injection of insulin (5 U iv), although the change in liver did not reach statistical significance (Fig. 6, H–J). These data indicate that SK1 deficiency improves systemic insulin sensitivity in DIO mice primarily by restoring adipose tissue and muscle insulin sensitivity.

Loss of SK1 ameliorates adipocyte proinflammatory responses and restores an anti-inflammatory phenotype. Adipose tissue inflammation initiated by inflammatory signals generated by adipocytes and propagated by infiltration and activation of immune cells, particularly macrophages, is a key factor in the development of insulin resistance and T2D. Although aspects of adipose inflammation are well understood, initial cues driving obesity-related adipocyte inflammation are unclear. To determine whether SK1/S1P signaling influences adipocyte inflammation in vivo, we measured the expression of inflammatory genes in adipocytes purified from DIO WT or SK1/−/− mice. Proinflammatory cytokines TNFα, IL-6, and

![Fig. 7. SK1 promotes adipocyte proinflammatory responses](http://ajpendo.physiology.org/)

**Fig. 7.** SK1 promotes adipocyte proinflammatory responses. TNFα (A), IL-6 (B), monocyte chemotactic protein-1 (MCP-1; C), plasminogen activator inhibitor-1 (PAI-1; D), IL-10 (E), adiponectin (F), and suppressor of cytokine signaling 3 (SOCS3; G) mRNA levels in adipocytes isolated from EAT of WT and SK1/−/− fed a HFD for 16 wk; n = 10 ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001, WT vs. SK1/−/−.
monocyte chemotactic protein-1 (MCP-1) were lower in EAT of SK1<sup>−/−</sup> compared with WT mice, but there was no difference in plasminogen activator inhibitor 1 mRNA levels (Fig. 7, A–D). The reduction in proinflammatory molecules was accompanied by a concurrent increase in expression of the anti-inflammatory mediator IL-10 (Fig. 7E) and adiponectin (Fig. 7F), known to protect from insulin resistance (28, 29). We also examined expression of suppressor of cytokine signaling 3 (SOCS3), which contributes to inflammation-driven insulin resistance (39, 40). Adipocytes from HFD-fed SK1-deficient mice had dramatically reduced SOCS3 mRNA levels compared with WT mice (Fig. 7G), providing additional mechanisms by which SK1 may contribute to insulin resistance. These findings are consistent with a model where adipocyte-derived S1P might act in concert with other adipocyte mediators (e.g., MCP-1) to facilitate ATM recruitment. In obese adipose tissue, an increase in the number of histological features known as crown-like structures (CLS) that represent accumulation of one or more macrophages around a single adipocyte correlate with adipose inflammation and insulin resistance. Although CLS-containing macrophages were readily observed surrounding adipocytes in the EAT of DIO WT mice (Fig. 8A), the number of CLS was markedly lower in EAT of DIO SK1<sup>−/−</sup> mice (Fig. 8, A–C). Moreover, transcription of the macrophage markers F4/80 and CD68 was signifi-

SK1 contributes to ATM recruitment and regulates the macrophage inflammatory phenotype. In the context of the total macrophage population in the adipose tissue, increased numbers of M1 (CD11b<sup>+/CD11c</sup>) macrophages that are primarily proinflammatory account for the majority of the increase in ATM in obesity, and >90% of recruited monocytes become CD11c<sup>+</sup> adipose tissue macrophages, and this is linked to insulin resistance (28, 29). Although the resident population of M2 (CD11b<sup>+/CD11c</sup>) macrophages is considered to be anti-inflammatory with homeostatic function, their origin and role in adipose function and insulin resistance are less understood. Because S1P is a potent chemoattractant for monocytes and macrophages (13), we hypothesized that adipocyte-derived S1P might act in concert with other adipocyte mediators to facilitate ATM recruitment. In obese adipose tissue, an increase in the number of histological features known as crown-like structures (CLS) that represent accumulation of one or more macrophages around a single adipocyte correlate with adipose inflammation and insulin resistance. Although CLS-containing macrophages were readily observed surrounding adipocytes in the EAT of DIO WT mice (Fig. 8A), the number of CLS was markedly lower in EAT of DIO SK1<sup>−/−</sup> mice (Fig. 8, A–C). Moreover, transcription of the macrophage markers F4/80 and CD68 was signifi-

---

**Fig. 8.** SK1/S1P signaling promotes adipose tissue macrophage recruitment during diet-induced obesity (DIO). Immunohistochemistry for F4/80 from sections of EAT from WT (A) and SK1<sup>−/−</sup> mice (B) showing macrophage staining within “crown-like structures” (CLS; arrows). C: quantification of CLS in EAT from HFD-fed WT and SK1<sup>−/−</sup> mice; n = 6 ± SD. D and E: macrophage marker F4/80 (D) and CD68 mRNA expression (E) in EAT of WT and SK1<sup>−/−</sup> mice; n = 10 ± SD. F and G: FACS analysis of CD11b<sup>+</CD11c</sup> macrophages in EAT SVF from DIO WT and SK1<sup>−/−</sup> mice. SVFs from 2 or 3 mice were typically pooled; n = 3. H: relative SK1 mRNA in adipocytes and macrophages from EAT of WT DIO mice. SK1/S1P signaling contributes to adipose macrophage inflammation. I–L: TNFα (I), IL-6 (J), MCP-1 (K), and IL-10 (L) mRNA levels in CD11c<sup>+</sup> macrophages isolated from EAT of WT and SK1<sup>−/−</sup> mice fed a HFD for 16 wk. For H–L, n = 5 ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, WT vs. SK1<sup>−/−</sup>.
cantly lower in adipose tissues of DIO SK1\(^{-/-}\) mice (Fig. 8, D and E). FACS analysis of SVF cells from these mice indicates that the population of M1 (CD11b\(^+/\)CD11c\(^+\)) macrophages was significantly reduced in EAT of SK1\(^{-/-}\) mice compared with WT mice (Fig. 8, F and G). With respect to the population of M2 (CD11b\(^+/\)CD11c\(^-\)) macrophages, although there appeared to be a modest decrease in this population in the SK1\(^{-/-}\) mice, this change was not significant (Fig. 8, F and G). Thus SK1 is a significant instigator of M1 macrophage recruitment observed in the adipose tissues in obesity and does not have a major role in the regulation of the M2 resident population.

Although ATMs were lower in SK1-null mice compared with their WT counterparts, whether SK1/S1P signaling contributes to its proinflammatory phenotype remains to be determined. This is potentially important since SK1 is expressed in monocytes and macrophages (34) and is also expressed in adipose tissue-derived M1 macrophages; however, its relative expression is modestly but insignificantly lower than in adipocytes (Fig. 8H). To determine the inflammatory phenotype in this population of macrophages, we analyzed expression of cytokine genes in the CD11c\(^+\) cell population isolated from adipose SVF from WT and SK1\(^{-/-}\) mice. SK1-deficient CD11c\(^+\) cells expressed lower levels of TNFα and IL-6 mRNA and higher levels of IL-10 mRNA than cells from WT mice (Fig. 8, I, J, and L). MCP-1 mRNA levels were similar in CD11c\(^+\) macrophages from WT and SK1\(^{-/-}\) mice (Fig. 8K). These results suggest that loss of SK1 induces an anti-inflammatory macrophage phenotype associated with protection from insulin resistance.

**Pharmacological inhibition of SK1 reduces adipose inflammation and improves glucose homeostasis in DIO mice.** To evaluate the potential of SK1 as a therapeutic target to improve glucose homeostasis, we treated mice with 5c, a small-molecule inhibitor of SK1 with documented effects in vivo (34). 5c is specific for the SK1 isofrom, does not inhibit SK2 or PKC, and shows no cytotoxicity at effective doses (30, 34). Inhibition of SK1 with 5c ameliorates endotoxin-induced proinflammatory cytokine production by phagocytes and protects mice from endotoxin shock (34). DIO WT mice were administered 5c (2 mg/kg ip) or vehicle once daily for 3 days (34), and glucose homeostasis was evaluated. This treatment regimen with 5c significantly improved glucose tolerance and insulin resistance (Fig. 9, A and B) and lowered fasting blood glucose levels (Fig. 9C) but had no effect on plasma FFA or triglyceride levels (not shown). In response to 5c treatment, insulin-mediated Akt phosphorylation was increased in adipose, liver, and muscle, although the increase in muscle did not reach significance (Fig. 9, D–F). These data suggest that acute pharmacological inhibition of SK1 in DIO mice improves systemic glucose homeostasis by increasing insulin-signaling pathways in these primary insulin target tissues. To determine whether these metabolically favorable changes were mechanistically related to decreased adipose inflammation, we analyzed the expression of key inflammatory markers in EAT-derived adipocytes and SVF cells from control and 5c-treated mice. In

![Graphs showing blood glucose levels, Akt phosphorylation in liver and muscle, and adipose tissue Akt phosphorylation](http://ajpendo.physiology.org/)

Fig. 9. Pharmacological inhibition of SK1 improves insulin sensitivity. GTT (A), ITT (B), and fasting glucose levels (C) in DIO WT mice treated daily for 3 days with specific SK1 inhibitor 5c (2 mg/kg ip) or with vehicle control. Insulin-mediated Akt phosphorylation in liver (D), muscle (E), and EAT (F) of control and 5c-treated DIO mice. The bands of vehicle and 5c-treated DIO WT mice in the Western blot are from the same blot and at the same exposure level. D–F, bottom: same blot stripped and reprobed with anti-Akt. Black line between some of the bands indicates noncontiguous lanes. The separating white space between control and 5c-treated samples denotes membrane splicing for clear presentation; \(n = 6 \pm SD. *P < 0.05\) and \(*P < 0.01, 5c\) vs. control.
adipocytes, specific inhibition of SK1 reduced TNFα, IL-6, MCP-1, and SOCS3 mRNA levels (Fig. 10, A–D) and concomitantly increased IL-10 and adiponectin mRNA levels (Fig. 10, E and F). In the SVF cells, which include ATM, SK1 inhibition suppressed TNFα and insignificantly reduced IL-6 transcription (Fig. 10, G and H) and increased IL-10 (Fig. 10J) but had no effect on MCP-1 gene expression (Fig. 10I). Collectively, these data show that the transcriptional profile of adipocytes and SVF cells from SK1 inhibitor-treated mice phenocopies that of adipocytes and ATM from SK1−/− mice, providing further support for a critical role for SK1 in obesity-induced adipose inflammatory responses that drive insulin resistance and T2D.

**DISCUSSION**

This study establishes a role for SK1 in obesity-mediated adipose dysfunction and insulin resistance. We found that genetic disruption of the SK1/S1P signaling pathway decreased adipocyte proinflammatory and chemotactic signals, attenuated ATM accumulation and inflammation, increased adipogenesis, decreased steatosis, and contributed to improved systemic insulin sensitivity. Similarly, pharmacological inhibition of SK1 in DIO WT mice shifted the adipose tissue milieu from proinflammatory to anti-inflammatory, resulting in upregulation of IL-10 and adiponectin, enhanced insulin sensitivity, and improved glucose homeostasis. SK1 and S1P are increased in adipose tissues of DIO mice. Our finding that SK1 mRNA is also increased in adipose tissues from obese T2D patients is clinically relevant and consistent with studies showing increased adipose S1P in obese humans (6) and high SK1 activity in humans with abnormally higher levels of adipose tissue inflammation and liver fat (21). Plasma S1P is also reported to be higher in obese T2D subjects (12). Compared with WT DIO mice, SK1-deficient DIO mice accumulated fewer ATM, and their adipocytes produced lower levels of TNFα, IL-6, MCP-1, and SOCS3 and higher levels of IL-10 and adiponectin. Moreover, specific pharmacological inhibition of SK1 in WT DIO mice increased IL-10 and adiponectin levels and decreased SOCS3. These cytokine responses are well-documented mechanisms that improve insulin sensitivity and overall glucose homeostasis (28, 29). Our findings that adipocyte and macrophage inflammatory responses are reduced in HFD-fed SK1−/− mice complement a growing body of literature implicating SK1/S1P signaling in inflammation, immune cell function, and disease (25, 41). The SK1-S1P axis contributes to Toll-like receptor 4 signaling (31, 41) and promotes activation of IKK and NF-κB (33, 41), responses that can directly inhibit insulin signaling (29). Our observation that ATM accumulation is reduced in DIO SK1−/− mice is consistent with decreased levels of MCP-1 in SK1−/− mice and established roles of SK1/S1P in trafficking and migration of numerous immune cells, including macrophages (13). Contribution of S1P to macrophage recruitment has been demonstrated in systems where apoptotic cells upregulate SK1 and secrete S1P, which directly attracts macrophages (13). Indeed, macrophages are found in CLS around hypertrophic and apoptotic adipocytes in obese mice and humans (10).

The insulin-sensitive phenotype of the HFD-fed SK1−/− mice, despite increased adiposity, is consistent with numerous studies indicating that overall adipose function and not adiposity per se contributes to metabolic dysfunction. For example, overexpression of adiponectin in obese mice led to adipose tissue expansion without inflammation and maintained insulin sensitivity despite profound obesity (20). Both obese mice deficient in macrophage galactose-type C-type lectin 1 (48) and mice with adipocyte-specific deletion of the nuclear corepressor NCoR (23) were protected from glucose intolerance, insulin resistance, and steatosis despite having more visceral fat. We found that increased adiposity in HFD-fed SK1−/− mice was related to increased adipogenesis, suggesting that adipose
SK1/SIP signaling may be a pathological signal that limits adipogenesis and during weight gain. These results are consistent with data indicating that the SIP analog FTY720 inhibits the differentiation of 3T3-L1 preadipocytes (27) but contrast with a study using siRNA-mediated SK1 knockdown in 3T3-L1 adipocytes, which concluded that SK1 enhances adipogenesis (15). In our in vivo studies using primary adipocytes, SK1 may better reflect the contribution of SK1/SIP to adipogenesis than cell lines in vitro. The mechanism of increased adipogenesis in our SK1−/− mice may be related to decreased phosphorylation of PPARγ on Ser112, which inhibits ligand binding by PPARγ and reduces its ability to promote adipogenesis (11).

Ceramide, which lies upstream of SIP, not only inhibits insulin signaling but also contributes to obesity via effects on energy expenditure and metabolism (17, 37). In contrast, the SK1-SIP signaling axis does not drive overall obesity per se, but plays a significant role in adipose inflammation leading to insulin resistance. In rat adipocytes, SIP increases lipolysis and suppresses insulin-induced leptin secretion, consistent with a role in promoting insulin resistance (18). However, Ma et al. (24) showed that adenoviral-mediated expression of human SK1 in KK/Ay T2D diabetic mice (Ad-SPHK1) reduced blood glucose levels and improved insulin sensitivity. In this instance, the effect of SK1 gene delivery on the tissue distribution of ceramide in these mice was not reported. SK1-derived SIP can negatively regulate ceramide levels in some systems (22, 26). Thus, increases in SK-1/SIP in Ad-SPHK1 mice could decrease levels of total ceramide or specific ceramide subspecies, which would explain the improved insulin sensitivity observed in this model. Consistent with this possibility, HFD-fed SK1 transgenic mice show reduced muscle insulin resistance associated with decreased levels of muscle ceramide but no change in SIP (8). Treating HFD-fed mice with the SIP analog FTY720 reduced muscle ceramide and improved glucose tolerance (9). In both of the above instances, the specific decrease in ceramide was also associated with reduced obesity. In the DIO SK1−/− mice studied here, decreases in SIP were not associated with changes in plasma or adipose tissue ceramide, suggesting that the improvements in adipose function and glucose homeostasis in this model are due to the decrease in SK1/SIP signaling.

Although mice where SK1 has been genetically deleted have revealed interesting potential actions of the SK1-SIP signaling axis in adipose inflammation and systemic insulin resistance, these may also represent potential secondary indirect effects mediated by downregulation of SK1/SIP in other tissues as well, particularly in insulin target tissues such as the liver and the muscle. Moreover, in the adipose tissue itself, the heterogeneity in terms of constituent cell types increases the challenge of determining cell-specific functions of SK1 in this tissue. In this respect, adipose tissue macrophages also express SK1, and proinflammatory gene expression was reduced in adipose CD11c+ macrophages from HFD-fed SK1−/− mice. Thus a role for adipose macrophage SK1/SIP signaling in modulating adipose inflammation also cannot be excluded. Generation of mice in which SK1 is ablated specifically in adipocytes or macrophages is needed to provide direct evidence of the functional significance of cell-specific SK1/SIP signaling to obesity-mediated adipose inflammation and systemic insulin resistance.

SIP signals via five G protein-coupled receptors (S1PR1–5). The effect of SIP on a given cell type is determined by the pattern and level of expression of its receptors, which are coupled to different downstream signaling cascades. Identification of specific S1PRs and downstream signaling pathways that regulate adipogenesis, ATM recruitment, and inflammation is currently being investigated in our laboratory.

In conclusion, our data indicate that the SK1-SIP axis contributes to impaired adipogenesis and adipose inflammation and to insulin resistance. Therefore, inhibition of SK1 merits consideration as a potential therapeutic strategy to treat or reduce insulin resistance, T2D, and the metabolic syndrome.

ACKNOWLEDGMENTS

We thank Dr. Richard Proia (NIH) for proving the SK1−/− mice. We thank Dr. Robert R. Henry (UCSD) for providing the human samples used in the study. We thank A. Samad for the preparation of figures.

GRANTS

This study was supported by NIH grants HL-71146 and HL-104232 (F. Samad), a grant from the Diabetes National Research Group (F. Samad), and grants from the Medical Research Service, Department of Veterans Affairs, Veterans Affairs San Diego Healthcare system (R. R. Henry), and the American Diabetes Association (T. P. Ciaraldi and R. R. Henry).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.W., L.B., J.B., T.P.C., and F.S. performed experiments; J.W., L.B., and T.P.C., and F.S. analyzed data; J.W., L.B., and F.S. interpreted results of experiments; J.W., L.B., and F.S. prepared figures; J.W., J.B., T.P.C., and F.S. approved final version of manuscript; T.P.C. and F.S. edited and revised manuscript; F.S. conception and design of research; F.S. drafted manuscript.

REFERENCES

9. Bruce CR, Risis S, Babb JR, Yang C, Lee-Young RS, Henstridge DC, Febbraio MA. The sphingosine-1-phosphate analog FTY720 reduces...
SPHINGOSINE KINASE 1 IN ADIPOSE INFLAMMATION


