Selective inhibition of sphingosine kinase-1 protects adipose tissue against LPS-induced inflammatory response in Zucker diabetic fatty rats

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Submitted 3 February 2014; accepted in final form 17 July 2014

Tous M, Ferrer-Lorente R, Badimon L. Selective inhibition of sphingosine kinase-1 protects adipose tissue against LPS-induced inflammatory response in Zucker diabetic fatty rats. Am J Physiol Endocrinol Metab 307: E437–E446, 2014. First published July 22, 2014; doi:10.1152/ajpendo.00059.2014.—Obesity is associated with a state of chronic inflammation. The chemokine (C-C motif) ligand 5 (CCL5) has been proposed to modulate the inflammatory response in adipose tissue (AT). However, the mechanisms underlying CCL5 upregulation in AT remain undefined. The objective of the present study was to evaluate whether the enzyme sphingosine kinase-1 (SK1) would modulate the expression of CCL5 and other inflammatory biomarkers in primary adipocytes and its potential role in lipopolysaccharide (LPS)-induced AT inflammation in a rat model of diabetes. To address this, LPS-stimulated primary adipocytes and 3T3-L1 cells were treated with a SK inhibitor, and the expression of Ccl5 and other CC chemokines were studied. Moreover, the effect of SK1 knockdown on cytokine production was analyzed in 3T3-L1 cells by transfection of SK1-specific small-interfering RNA (siRNA). The anti-inflammatory effects of SK inhibitor in AT were also investigated in vivo using the Zucker lean normoglycemic control (ZLC) rats. LPS treatment stimulated Ccl5, IL-6, pentraxin 3 (Ptx3), and Tnfα mRNA expression in primary adipocytes and 3T3-L1 cells, whereas pharmacologically and siRNA-mediated SK1 inhibition strongly reduced mRNA levels of proinflammatory cytokines in these cells. Similarly, administration of SK inhibitor to ZLC rats prevented the LPS-induced inflammatory response in AT. Our data demonstrate a role for SK1 in endotoxin-induced cytokine expression in adipocytes and suggest that inhibition of SK1 may be a potential therapeutic tool in the prevention and treatment of chronic and common metabolic disorders, including obesity, insulin-resistance, and type 2 diabetes.

lipopolysaccharide; adipose tissue; chemokine (C-C motif) ligand 5; inflammation; sphingosine kinase-1; Zucker diabetic fatty rats

INFLAMMATION IS A COMMON COMPONENT of the pathogenesis of obesity, insulin resistance (IR), and type 2 diabetes (5, 9, 29, 32, 34). Several studies in animal models have shown the importance of macrophage infiltration into adipose tissue (AT) in obesity-induced IR (5, 29, 32), resulting in systemic IR and ultimately the development of type 2 diabetes. AT in obesity shows increased numbers of CD3-positive cells (25, 34). Infiltration of cytotoxic T cells [also known as cytotoxic T lymphocytes (CTL)] into obese AT is thought to precede macrophage accumulation (15, 21). T cell-derived cytokines such as interferon (IFN)–γ promote the recruitment and activation of M1 macrophages, increasing inflammation and IR in AT (26, 28, 35).

Among others, the chemokine (C-C motif) ligand 5 (CCL5), also known as RANTES (regulated on activation normal T cell expressed and secreted), has been identified as an important mediator for the recruitment of inflammatory cells (CTL and monocytes) into AT (12, 14, 25). CCL5, a 7.8-kDa CC chemokine, was initially considered a T cell-specific protein but has since been found to be produced by numerous cell types, including fibroblasts, endothelial cells, platelets, and monocytes/macrophages. Ccl5 mRNA levels are strongly associated with CD3- and CD11b-positive cells in human visceral adipose tissue. Subcutaneous white adipose tissue (sc WAT) mRNA abundance of Ccl5 and its receptor Ccr5 [chemokine (C-C motif) receptor 5] is significantly increased in obese humans with metabolic syndrome (34). Furthermore, in obese patients, the expression of the CC chemokines (CCL2, CCL3, CCL5, CCL7, CCL8, and CCL11) in AT and their receptors (CCR1, CCR2, CCR3, and CCR5) has been found to correlate with CD68 expression, a macrophage marker (10). Circulating levels of CCL5 have been reported to be elevated in patients with impaired glucose tolerance and type 2 diabetes (15, 22). Previous gene knockout studies have demonstrated conclusively that CCR5 is essential for the recruitment and polarization of AT macrophages (ATMs) (16). These results highlight the importance of the CCR5/CCL5 axis in the recruitment of inflammatory cells to AT and, consequently, in the initiation and progression of its inflammatory response. Nevertheless, it is still not known which mechanisms will trigger upregulation of CCL5 in WAT of obese subjects. Further studies are needed to better elucidate the molecular mechanisms underlying upregulation of key adipokines in WAT of obese subjects with metabolic syndrome that prompt the onset of type 2 diabetes.

Using the Zucker diabetic fatty (ZDF) (Lep(ob)fa/ fa) rat, an animal model of obesity and diabetes that shows characteristics similar to metabolic syndrome in humans (2), we hypothesized that adipocyte-expressed CCL5 plays an important role in WAT inflammation and that the enzyme sphingosine kinase-1 (SK1), which has been demonstrated to be involved in the inflammatory response in leukocytes (11, 13, 36), would modulate the expression of CCL5 and other proinflammatory cytokines in WAT contributing to the progressive metabolic complications of obesity.

The aim of this study was to address whether the enzyme SK1 might play a role in WAT inflammation and whether SK1 downregulation by siRNA or using SK inhibitors might prevent the LPS-induced inflammatory response in WAT in ZDF rats.
MATERIALS AND METHODS

**Rats.** Seven-week-old male ZDF rats and their lean littersmates [Zucker lean normoglycemic control (ZLC) rats] (Lepr<sup>db/db</sup>-Lepr<sup>+/+</sup>) were purchased from Charles River Laboratories (Wilmington, MA). Rats were fed standard rodent Chow diet (LabDiet 5008; Panlab, Barcelona, Spain) and given water ad libitum in a controlled environment with a 12:12-h light-dark cycle. Rats were subjected to two different protocols. In protocol 1, 12-wk-old ZDF and ZLC rats (n = 6–8/group) were fasted 6 h before euthanization, and then animals were anesthetized and euthanized by cardiac puncture. After blood collection, sc WAT was immediately dissected and frozen in liquid nitrogen and stored at −80°C or processed for preadipocyte isolation. In protocol 2, 12-wk-old ZLC rats were randomly divided into one of the following treatment groups (n = 4/group): saline (group 1), LPS (group 2, 10 mg/kg body wt, LPS from *Escherichia coli* 055:B5; Sigma-Aldrich), St. Louis, MO) or LPS (10 mg/kg body wt) combined with SK inhibitor (group 3, 10 mg/kg body wt; Calbiochem, La Jolla, CA), respectively. Sixty minutes prior to LPS injection, rats received a single dose of saline (groups 1 and 2) or SK inhibitor (group 3) (ip). Rats were euthanized 18 h after saline or LPS administration (and fasted 6 h before euthanization), and sc WAT was obtained for mRNA and protein extraction. All procedures followed in the study fulfilled the criteria of the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (Publication No. 85-23, revised 1996) and were approved by the Internal Animal Committee Review Board of Hospital de la Santa Creu i Sant Pau.

**Isolation of preadipocytes and differentiation to adipocytes.** Subcutaneous WAT from ZDF and ZLC rats (n = 6–8/group) was washed and digested with collagenase type I (Sigma-Aldrich) and trypsin (5 mg/ml) and subcultured in six-well plates (Corning). All preadipocytes used in our study were cultured for three passages before differentiation. Two-day postconfluent preadipocytes (passage 3) were induced to differentiate in differentiation induction medium [1 μM dexamethasone, 0.5 mM IBMX, 0.1 mM indomethacin, and 10 μg/ml human insulin (Sigma-Aldrich)] in DMEM-F12 (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% (vol/vol) FBS (Biological Industries, Kibbutz Beit-Haemek, Israel) and 1% (vol/vol) penicillin-streptomycin (Gibco, Life Technologies). Cells were induced to differentiate in differentiation induction medium [1 μM dexamethasone, 0.5 mM IBMX, 0.1 mM indomethacin, and 10 μg/ml human insulin (Sigma-Aldrich)] in DMEM-F12 (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% (vol/vol) FBS (Biological Industries, Kibbutz Beit-Haemek, Israel) and 1% (vol/vol) penicillin-streptomycin (Gibco, Life Technologies). Cells were fed every 2 days, and on day 21 >90% cells had differentiated to adipocytes. Light microscopy and Oil Red O staining were used to monitor the characteristic cell rounding and lipid droplet accumulation in these cells during differentiation. Furthermore, several genes involved in lipid metabolism (genes involved in modulating the mature adipocyte phenotype) were studied in both preadipocytes and differentiated adipocytes (on day 21). To study the LPS-induced inflammatory response, mature adipocytes were serum starved for 16 h and then pretreated with either SK inhibitor SPHK I (10 μM; Calbiochem) or vehicle and stimulated with 1 μg/ml LPS (LPS from *Escherichia coli* 055:B5; Sigma Aldrich) for 4 or 72 h (for isolation of RNA or ELISA determinations, respectively).

**Cell culture.** 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 10% (vol/vol) calf serum and antibiotics (1% penicillin-streptomycin) at 37°C in 5% CO<sub>2</sub> in air. Two days postconfluence (day 0), preadipocytes were induced to differentiate in a standard differentiation induction medium (0.5 mM IBMX, 1 μM dexamethasone, and 1 μg/ml human insulin) in DMEM, 10% (vol/vol) FBS, and 1% (vol/vol) penicillin-streptomycin. Two days later (day 2), this medium was replaced with DMEM and 10% FBS (vol/vol) containing 1 μg/ml human insulin. After day 4, medium was replaced with DMEM and 10% FBS (vol/vol), and studies were performed on days 7–10. 3T3-L1 preadipocytes or mature adipocytes were serum-starved for 16 h and then pretreated with the SK inhibitor SPHK I (Calbiochem) or SK-I (Tocris Bioscience, Bristol, UK), and 60 min later, cells were treated with either 1 μg/ml of LPS or palmitate (PAL; Sigma-Aldrich). PAL solutions (final concentration, 1 mM) were administered to cells as a conjugate with fatty-acid-free BSA (2% fatty acid free-BSA) for 8 or 72 h (for isolation of RNA or ELISA determinations, respectively).

**Quantitative real-time PCR.** Total RNA was extracted from WAT by the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed with 1 μg of total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out using the SYBR Green I assay and the 7900 Sequence Detection System (Applied Biosystems). Rat and mouse designed primers are listed in Tables 1 and 2, respectively. Cd68, Cd163, Tf educate mRNA expression in sc WAT, and Ccl5 and SKI mRNA expression in transfected-3T3-L1 adipocytes were examined by quantitative real-time PCR using TaqMan Assays-on-Demand (Applied Biosystems). Values were normalized to the ribosomal protein large P0 (Rplp0) expression that was stable in the samples measured.

**Silencing the SK1 gene.** 3T3-L1 cells were allowed to differentiate, following the previously described protocol. Mature adipocytes were transfected using RNAiMAX Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The predesigned small-interfering RNA (siRNA) specific to SK1 (Silencer Select siRNA) and control siRNA (Silencer Select Control siRNA) were purchased from Ambion (Applied Biosystems).

Table 1. List of rat primers used for real-time PCR

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Cc2 and -5, chemokine (C-C motif) ligand 2 and 5, respectively; Ccr1, -3, and -5, chemokine (C-C motif) receptor 1, 3, and 5, respectively; Lpl, lipoprotein lipase; Ptx3, pentraxin 3; Rplp0, ribosomal protein large P0; SK1 and -2, sphingosine kinase 1 and 2, respectively.

*AJP-Endocrinol Metab* • doi:10.1152/ajpendo.00059.2014 • www.ajpendo.org
Table 2. List of mouse primers used for real-time PCR

<table>
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Fabp-4, fatty acid-binding protein-4.

ELISA. CCL5 concentrations in serum samples, sc WAT, and cell supernatants were determined using the rat RANTES ELISA Development Kit (Peprotech, Rocky Hill, NJ) according to the manufacturer's protocol.

SK activity. Activity of SK1 and SK2 was measured as phosphorylation of ω-(7-nitro-2,1,3-benzoxadiazol-4-yl)-n-erythro-sphingosine (NBD-sphingosine; Avanti Polar Lipids, Alabaster, AL) to NBD-sphingosine-1-phosphate (S1P), as described previously (4). Cell migration assays were performed in a transwell chamber, using Transwell inserts with a 5-μm porous membrane (Corning). THP-1 cells (human acute monocytic leukemia cell line; American Type Culture Collection) were placed in the upper chamber (Corning). THP-1 cells (human acute monocytic leukemia cell line; American Type Culture Collection) were placed in the upper chamber (Corning). THP-1 cells were maintained in the lower chamber using a Neuhuaber chamber.

Statistical analyses. Results are expressed as means ± SD. Significant differences among groups were analyzed using the Student’s t-test (to compare 2 groups) or one-way ANOVA (data involving more than 2 groups). Two-way repeated-measures ANOVA was used to assess differences among time points and between groups in time course studies performed in 3T3-L1 adipocytes. Data were analyzed using GraphPad Prism (La Jolla, CA) software version 4. Differences were considered significant at P ≤ 0.05.

RESULTS

Different expression patterns of CCL5 and its receptors in sc WAT of ZDF and ZLC rats. Biochemical characteristics of ZLC and ZDF rats at baseline are presented in Table 3. As shown in Fig. 1A, Ccl5 mRNA levels studied in sc WAT were increased significantly in ZDF rats compared with controls (P < 0.005). The increased Ccl5 gene expression in sc WAT-ZDF rats was also evident at the protein level (compared with control rats) (P < 0.01; Fig. 1B), whereas CCL5 serum levels were not different between ZDF and control lean rats (856.5 ± 47.9 vs. 582.1 ± 124.3 pg/ml; Fig. 1C). Also, higher Ccr1 and Ccr5 mRNA levels were observed in sc WAT-ZDF rats (P < 0.005; Fig. 1, D and F), whereas the expression of Ccr3 in AT did not differ between groups (Fig. 1E).

LPS-stimulated adipocytes obtained from ZDF rats express and secrete higher levels of CCL5 than those obtained from ZLC rats. Differentiated adipocytes exhibited changes in mRNAs encoding markers of adipocyte differentiation (Fig. 2A) that were consistent with morphological differences (Fig. 2, D–F). As shown in Fig. 2B, Ccl5 mRNA levels were barely detectable in unstimulated cultured preadipocytes and were significantly higher than those found in mature adipocytes (P = 0.001). Cell supernatants collected after 72 h in starving DMEM-F-12 medium showed low levels of CCL5 released from unstimulated cultured preadipocytes and were significantly lower than those found in mature adipocytes (P < 0.005; Fig. 2B). Importantly, Ccl5 mRNA levels in mature adipocytes were significantly higher than those found in preadipocytes (P < 0.005; Fig. 2B). Notably, in mature adipocytes, Ccl5 expression induced by LPS was significantly higher in cells derived from ZDF rats compared with ZLC rats (P < 0.05; Fig. 2B). Results from ELISA analysis confirmed that LPS significantly induced CCL5 secretion by adipocytes (compared with control cells, P < 0.005; Fig. 2C). Again, mature adipocytes from ZDF rats secreted higher amounts of CCL5 than those from ZLC rats (553.2 ± 28.3 vs. 347.3 ± 55.3, P < 0.05; Fig. 2C).

LPS induces SK1 activation and its expression in mature adipocytes. Many studies have demonstrated that monocyte/macrophage infiltration and levels of TNFα and other proinflammatory cytokines could be mediated by SK1. To determine the role of SK1 in CCL5 expression and secretion by primary rat adipocytes, mature adipocytes (obtained from ZDF and ZLC rats) were pretreated with the SK inhibitor SPHK I (10 μM) for 60 min and exposed to LPS for 0.5, 2, and 4 h, and SK1/SK2 activity and mRNA levels were analyzed. Results showed that LPS triggers SK1 activation in rat mature adipocytes, whereas SK2 activity is not affected (data not shown). SK1 mRNA expression was significantly induced after 4 h of LPS treatment (P = 0.01; Fig. 3A), whereas pretreatment of cells with SPHK I blocked LPS-induced SK1 mRNA expression (P < 0.05; Fig. 3A). In contrast, expression of SK2 was not induced by LPS stimulation (Fig. 3B). These data suggest...
LPS-induced adipose cells. As shown in Fig. 4, the expression of other proinflammatory cytokines was also tested on primary adipocytes. Data from collections at 72 h after LPS stimulation confirmed these results. Secreted CCL5 levels (ELISA) in supernatants collected from differentiated adipocytes of ZDF and ZLC rats (Fig. 4). SK2 is not involved in the induction of CCL5 in adipose cells. As we had seen in primary rat adipocytes, pretreatment with SPHK I (10 μM) significantly reduced LPS-induced CCL5 chemokine released in the media in both preadipocytes and mature adipocytes (P < 0.001; Fig. 5, C and D).

Pharmacological inhibition of SK1 decreases the expression of CCL5 and other proinflammatory cytokines in rat adipose cells. SPHK I substantially reduced CCL5 chemokine released in the media of ZDF rats (compared with control ZLC rats; Fig. 5). Although treatment with PAL significantly induced CCL5 secretion by adipose cells and the SK1 inhibitor decreased PAL-induced CCL5 secretion (Fig. 5), SKPH I was not able to decrease the CCL5 mRNA levels measured in mature adipocytes 8 h after stimulation (P < 0.05; Fig. 6).

SK1 silencing provided a 71% reduction in target mRNA levels in 3T3-L1 mature adipocytes (P < 0.0001; Fig. 6A). Furthermore, in SK1-transfected-cells, activity was not induced by LPS [compared with cells transfected with random scrambled control siRNA (si-control), P < 0.05; Fig. 6B]. As shown in Fig. 6C, CCL5 mRNA levels of cells transfected with specific siRNA were significantly lower than those found in cells transfected with si-control after 4 h of LPS stimulation (P < 0.0001). To further confirm that the SKPH1 isoform is involved in LPS-induced CCL5 mRNA expression, we used a potent isoenzyme-specific inhibitor of SK1 (SK1-I; 10 μM). SK1-I-treated cells showed a significant decrease in CCL5 mRNA expression compared with LPS-stimulated 3T3-L1 adipocytes (P < 0.001; Fig. 6D), and SK1-overexpressing 3T3-L1 cells displayed significantly higher CCL5 mRNA levels than the vector control cells after stimulation with LPS (data not shown). Taken together, our results indicated that the SK1 isoform is responsible for LPS-induced CCL5 upregulation in adipocytes.

SK1 activity correlates with CCL5 protein levels in sc WAT. Having confirmed that SK1 is involved in the expression of many cytokines by adipose cells, we next investig...
gated the role that SK1 could play in adipose tissue inflammation. SK1 mRNA expression, evaluated in sc WAT, was significantly higher in ZDF rats relative to control lean rats (P < 0.01; Fig. 7A). Also, SK1 activity was increased significantly in sc WAT of ZDF rats (P < 0.05; Fig. 7B). Furthermore, we observed a positive and significant correlation between SK1 activity and CCL5 protein levels in sc WAT (r² = 0.4542, P = 0.042; Fig. 7C), indicating that SK1 could be involved in the expression of CCL5 in WAT and consequently could influence and regulate the inflammatory response in this tissue.

Decreased expression of Ccl5 and other proinflammatory genes in sc WAT of SPHK I-treated rats. To gain further insight into the role that SK1 might play in the modulation of the inflammatory response in WAT, the effect of pharmacological inhibition of SK1 was determined in vivo using ZLC rats. As shown in Fig. 8A, LPS administration increased Ccl5 mRNA levels significantly in sc WAT from ZLC rats (P ≤ 0.001). CCL5 serum concentrations were significantly reduced after 18 h of LPS administration in sc WAT (806.0 ± 4.3 vs. 418.3 ± 169.7 pg/ml, P < 0.05; data not shown). Importantly, a single dose of SPHK I reduced Ccl5 mRNA expression in sc WAT (P < 0.05; Fig. 8A).

Fig. 2. A: expression of the adipogenic marker genes (Cd36, Lpl, and AdipoQ) in primary rat preadipocytes and mature adipocytes (on day 21) before stimulation experiments. In all of these experiments, mRNA levels were normalized to that of Rplp0, a gene whose expression is unaffected by adipogenesis. Open bars, preadipocytes obtained from sc WAT-ZLC/ZDF rats; black bars, mature adipocytes obtained from sc WAT-ZLC/ZDF rats. Data are expressed as means ± SD (n = 6–8 rats/group). *P < 0.001 vs. preadipocytes. B and C: LPS-induced CCL5 levels in adipocytes of ZDF rats (compared with ZLC rats); Ccl5 gene expression in primary preadipocytes and mature adipocytes after LPS stimulation (1 μg/ml); B) and CCL5 secretion (pg/ml) by mature adipocytes treated with DMEM-F-12 only (control) or DMEM-F-12 and LPS (1 μg/ml); C). Open bars, preadipocytes/mature adipocytes obtained from sc WAT-ZDF rats; black bars, preadipocytes/mature adipocytes obtained from sc WAT-ZDF rats. Data are expressed as means ± SD (n = 6–8 rats/group). *P < 0.05 vs. ZLC rats; **P < 0.005 vs. control group; ***P = 0.001 vs. preadipocytes. D–F: representative images of primary adipose cells before and after adipogenic differentiation. Rat primary preadipocytes isolated from sc WAT of ZLC rats on day 0 (undifferentiated cells; original magnification ×10 in D) and rat mature adipocytes on day 21 (original magnification ×10 and ×20, respectively, in E and F).

Fig. 3. LPS-induced sphingosine kinase 1 (SK1) mRNA levels in mature adipocytes. SK1 (A) and SK2 mRNA expression (B) measured in mature adipocytes after LPS stimulation (ZDF and ZLC rats). Data are expressed as means ± SD (n = 6–8 rats/group). *P < 0.05; **P = 0.01 vs. LPS group.
SHPK I also decreased mRNA expression of several AT proinflammatory markers significantly induced by LPS, such as *Cd68* and *Cd163*, *Il-6*, *Ccl2*, and *Tnf* (*P* < 0.05; Fig. 8, B–F).

LPS-induced migration of THP-1 cells was significantly inhibited in the presence of the anti-CCL5 antibody. Next, to test the effects of CCL5 (secreted by adipocytes) on the recruitment of inflammatory cells to AT, THP-1 cells were induced to migrate using supernatants collected from mature adipocytes stimulated with LPS and treated or not with SPHK I (10 μM). Supernatants from LPS-treated adipocytes significantly induced migration of THP-1 cells (*P* < 0.001; Fig. 8G), whereas SPHK I pretreatment, which led to lower (nonsignificant) levels of CCL5 released in the media, slightly decreased monocyte migration. Finally, a CCL5 antibody was added to the supernatants collected from LPS-stimulated adipocytes, and the effect on THP-1 migration was tested. As shown in Fig. 8G, CCL5-blocked supernatants of LPS-stimulated rat adipocytes significantly inhibited THP-1 cell migration (*P* < 0.001).

**DISCUSSION**

To our knowledge, this is the first report showing the role of SK1 in the LPS-induced cytokine expression in adipose tissue of ZDF rats.

SK catalyzes S1P biosynthesis from sphingosine (18). Two isozymes of SK (SK1 and SK2) have been cloned (17, 19). Although they generate the same product, both enzymes have opposing biological functions (20). Several proinflammatory stimuli have been reported to activate SK1 on human neutrophils and macrophages, and blockade of SK1 inhibits proinflammatory responses triggered by these stimuli (11, 13, 36). Recently, it has been described that SK1 regulates IL-6 expression in skeletal muscle of obese mice by exogenous PAL (27). However, it remains unclear whether SK1 expressed by adipocytes could initiate and/or exacerbate the inflammatory response in AT of obese subjects with metabolic syndrome. To address these issues, we used the ZDF rat, an animal model of obesity and diabetes. Both real-time PCR and SK1/SK2 activity assays revealed that a proinflammatory stimulus, LPS or PAL, increases *SK1* mRNA expression and triggers the activation of the enzyme SK1 in adipocytes, whereas *SK2* expression and activity remain unaltered. In the current study, we showed for the first time that activation of SK1 in both primary rat adipocytes and 3T3-L1 cells leads to the upregulation of numerous proinflammatory factors, including CCL5. Furthermore, inhibition of SK1 by either a chemical inhibitor (SPHK I) or SK1-specific small siRNA revealed that SK1 plays a crucial role in LPS-induced *Ccl5* expression in mature adipocytes. In this work we have used two types of SK inhibitors: the SPHK I, a nonlipid, small-molecule inhibitor that is not specific for SK1 or SK2, and a sphingosine analog (SK1-I) that displays specificity for SK1. The use of SK1-I, which has been described as a potent isoenzyme-specific inhibitor of SK1 (SK1-I), and the studies performed in SK1-overexpressing 3T3-L1 cells confirmed the results obtained in SPHK I-treated adipocytes.
Our findings are consistent with recent studies showing reduced levels of proinflammatory cytokines and increased levels of anti-inflammatory molecules such as adiponectin and IL-10 in adipose tissue of SK1-deficient mice (31).

Our results showed that SK1 activity and Ccl5 mRNA levels were significantly higher in primary mature adipocytes than in preadipocytes. Consistent with our results, it has been reported that SK1 is upregulated during adipogenesis in mouse 3T3-L1 cells (33) and that mature adipocytes secrete higher CCL5 levels than preadipocytes after LPS stimulation (23).

Previous studies have involved CCL5 in the proinflammatory response in human AT, leading to monocyte/lymphocyte recruitment (35). CCL5 interacts with three specific G protein-coupled receptors, CCR1, CCR3, and CCR5, expressed by leukocytes. CCR1 has been shown to be differentially expressed by inflammatory cells and that the higher mRNA levels of Ccr1 and Ccr5 found in AT of ZDF rats would prompt a more pronounced inflammatory response in these animals. Accordingly, a previous study reported higher levels of CCR5 (+) ATM in WAT of genetically and high-fat diet-induced obese mice (compared with control mice) and that Ccr5−/− mice are protected from insulin resistance, glucose tolerance, and hepatic steatosis (16).

Using double-knockout models would allow us to verify the role that both receptors play in the pathogenesis of obesity, IR, and type 2 diabetes mediated by CCL5.

Because of the contribution of SK1 in regulating Ccl5 gene expression in primary cultured adipocytes and 3T3-L1 cells, we next hypothesized that SK1, expressed by adipocytes, would play a role in the regulation of the inflammatory response in WAT of ZDF rats. Importantly, we have found that SK1 gene expression and SK1 activity were significantly increased in sc WAT of ZDF rats compared with controls. The higher activity of this enzyme found in sc WAT of ZDF rats is in line with previous results that have reported increased SK1 activity in sc WAT derived from obese mice (ob/ob) mice (6) and higher SK1 mRNA levels in AT from obese patients with type 2 diabetes (31).

We found that CCL5 expressed in sc WAT was positively associated with SK1 activity. Using an adipocyte-specific SK1-knockout model would help us to determine whether SK1 expressed by adipocytes has a major role in the regulation of the expression of CCL5 and consequently in the initiation and modulation of the inflammatory response in AT.

Fig. 5. SK1 modulates LPS-induced CCL5 expression in 3T3-L1 preadipocytes and mature adipocytes. A: Ccl5 mRNA levels measured in 3T3-L1 preadipocytes and mature adipocytes pretreated or not with SPHK I (10 μM) and stimulated with LPS (1 μg/ml). Data are expressed as means ± SD (n = 3–8/group); *P < 0.05 vs. control group; #P < 0.05 vs. control group; ##P < 0.001 vs. control group. C: Ccl5 mRNA levels measured in 3T3-L1 preadipocytes and mature adipocytes pretreated with SPHK I and stimulated with LPS (SPHK I + LPS group). C and D: 3T3-L1 preadipocytes (C) and mature adipocytes (D) were pretreated or not with SPHK I (10 μM) and stimulated with LPS (1 μg/ml) for 4, 6, 18, 24, 48, or 72 h, followed by the quantification of CCL5 levels in collected supernatants. *P < 0.001 vs. control group; #P < 0.05 vs. cells pretreated with SPHK I and stimulated with LPS (SPHK I + LPS group). E: CCL5 and IL-6 mRNA levels of 3T3-L1 mature adipocytes 8 h after palmitate (PAL) stimulation. *P < 0.05 vs. control group; *P < 0.05 vs PAL-treated cells (1 mM). F: CCL5 levels were measured in supernatants collected 48 h after PAL treatment (1 mM). *P < 0.05 vs. PAL-treated cells.
LPS is recognized by the innate immune system via the Toll-like receptor 4 and contributes to the inflammatory response in AT of mice and ZDF rats (30). Circulating levels of LPS in type 2 diabetes patients are increased (5). High-fat feeding and obesity result in increased levels of LPS, promoting systemic inflammation and metabolic dysfunction in mice (34).

Previous studies have demonstrated that proinflammatory responses associated with endotoxin are regulated by SK1 (24). Our results clearly demonstrate that a single dose of LPS (10 mg/kg) increased \( Ccl5 \) mRNA expression in WAT of ZLC rats and that blockade of SK resulted in lower \( Ccl5 \) mRNA transcript levels and decreased expression of two specific markers of monocytes/macrophages (\( Cd68 \) and \( Cd163 \)). Also, SK inhibition reduced \( IL-6 \), \( Ccl2 \), and \( TNF \) mRNA levels in AT. Thus, these results confirm a role for SK1 in the regulation of the key proinflammatory mediators induced by endotoxin in AT.

Obese individuals frequently have increased circulating levels of free fatty acids. In this study, to further explore the effects of SK1 on the proinflammatory response in AT, we also investigated the role that SK1 might play on the PAL-induced inflammation in AT. Importantly, our results clearly demonstrate that in mature adipocytes PAL activated SK1, leading to higher \( IL-6 \) mRNA and CCL5 protein levels. Consistent with our results, in a recent study, PAL treatment has been demonstrated to induce \( IL-6 \) expression in a SK1-dependent manner (27). Our findings suggest that activation of SK1 by LPS and free fatty acids could initiate and exacerbate a proinflammatory response in AT of obese individuals. Thus, SK1 inhibitors could represent an effective therapeutic tool in combating AT chronic inflammation. Identification of the role of SK1 in AT inflammation is an essential approach to identify potential therapeutic targets for the treatment of obesity-related complications.

Fig. 6. SK1, \( Ccl5 \) gene expression, and SK1 activity in 3T3-L1-differentiated adipocytes transfected with SK1-specific siRNA (si-SK1) and random scrambled control siRNA (si-control). A–C: SK1 gene expression (A), SK1-specific activity (B), and \( Ccl5 \) gene expression (C) measured in normal (si-control) and SK1 knockdown (si-SK1) 3T3-L1 cells exposed for 4 h to LPS (1 \( \mu g/ml \)), as indicated. Open bars, si-control 3T3-L1 cells; black bars, si-SK1 3T3-L1 cells. * \( P < 0.05 \); ** \( P < 0.0001 \) vs. si-control cells. D: \( Ccl5 \) mRNA expression was evaluated after 4 h of LPS stimulation in 3T3-L1 cells pretreated or not with the specific SK1 inhibitor SK1-I (10 \( \mu M \)). Data are expressed as means ± SD (\( n = 3–4 \) group). * \( P < 0.001 \); ** \( P < 0.0001 \) vs. LPS group.

Fig. 7. SK1 activity correlates with CCL5 protein levels in sc WAT. A and B: SK1 gene expression (A) and SK activity (B) measured in sc WAT from ZLC and ZDF rats (\( n = 4–6 \) rats/group). Open bars, sc WAT from ZLC rats; black bars, sc WAT from ZDF rats. Data are expressed as means ± SD. * \( P < 0.05 \); ** \( P < 0.01 \) vs. ZLC rats. C: correlation between CCL5 levels and SK1 activity in adipose tissue (\( r^2 = 0.4542, P = 0.042 \)). ●, CCL5 protein levels and SK1 activity obtained in sc WAT.
other risk factors that could activate SK1 in WAT should be in the objective of further studies to gain more insight into the mechanisms involved in the regulation of the inflammatory responses in AT.

In conclusion, we demonstrate that both LPS- and PAL-induced adipokine and inflammatory cytokine expression in AT is triggered by the activation of SK1 in adipocytes, which could result in an efficient leukocyte recruitment and further cytokine secretion. We also provided evidence that suppression of SK1 lowers the expression of CCL5 and other proinflammatory cytokines in AT. It is suggested that blocking SK1 activity might be a potential new treatment approach to prevent and control the inflammatory response in AT that is implicated in the onset and in the progression of type 2 diabetes and its complications.

ACKNOWLEDGMENTS

We thank Sonia Veiga and Maria Teresa Bejar for assisting with adipose tissue collection, isolation, and culture of primary adipose cells.

GRANTS

This work was funded by a Merck Serono Research Grant to M. Tous from the Fundación Salud 2000 (Clinical Research in Cardiometabolism, 2009), the Spanish Ministry of Health-Instituto de Salud Carlos III (RETIC RIC RD12/0042/0027 and RETIC TERCER RETIC RECAVA RD12/0019/0026), the Spanish Ministry of Science and Innovation (PNS SAF2013/42962-R), and Fundación de Investigación Cardiovascular-Fundación Jesús Serra. M. Tous was the Juan de la Cierva investigator (MICINN Program, JCI-2007-163-319) and Postdoctoral Fellow from Fundación de Investigación Cardiovascular (Barcelona). R. Ferrer-Lorente is a Sara Borrell Investigator (Instituto de Salud Carlos III).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M.T. conception and design of research; M.T. and R.F.-L. performed experiments; M.T. analyzed data; M.T. and L.B. interpreted results of experiments; M.T. prepared figures; M.T. drafted manuscript; M.T., R.F.-L., and L.B. edited and revised manuscript; M.T., R.F.-L., and L.B. approved final version of manuscript.
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