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Sphingosine Kinase 1 Knockdown Reduces Insulin Synthesis and Secretion in a Rat Insulinoma Cell Line

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Abstract

To evaluate the role of sphingosine kinase 1 (SphK1) in insulin secretion, we used stable transfection to knock down the expression of the Sphk1 gene in the rat insulinoma INS-1 832/13 cell line. Cell lines with lowered Sphk1 mRNA expression and SphK1 enzyme activity (SK11 and SK14) exhibited lowered glucose- and 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) plus glutamine-stimulated insulin release and low insulin content associated with decreases in the mRNA of the insulin 1 gene. Overexpression of the rat or human Sphk1 cDNA restored insulin secretion and total insulin content in the SK11 cell line, but not in the SK14 cell line. The Sphk1 cDNA-transfected SK14 cell line expressed significantly less SphK1 activity than the Sphk1 cDNA-transfected SK11 cells suggesting that the shRNA targeting SK14 was more effective in silencing the exogenous rat Sphk1 mRNA. The results indicate that SphK1 activity is important for insulin synthesis and secretion.

Keywords

Insulin secretion; insulin gene transcription; insulinoma INS-1 cells; gene knockdown; shRNA; siRNA; phospholipids; sphingosine-1-phosphate (S1P); sphingosine kinase (SphK); sphingosine; ceramide

1.0 Introduction

Sphingosine kinase isoforms 1 and 2 (SphK1 and SphK2) are lipid kinases that catalyze phosphorylation of sphingosine to generate sphingosine 1-phosphate (S1P) [1, 2]. Activation

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of SphK1 involves phosphorylation and translocation to the plasma membrane where its substrate sphingosine resides [3-5]. S1P is a bioactive sphingolipid metabolite that regulates a variety of cellular processes [1, 2]. It acts both as an intracellular second messenger and as a ligand for G protein-coupled S1P receptor isoforms 1 to 5 on the cell surface [6, 7] that regulate diverse signal transduction pathways and elicit pleiotropic responses unique to the type of cell [2]. Upon binding to its receptors, extracellular S1P can generate numerous cellular responses including differentiation, growth, survival, cytoskeleton rearrangements, cell motility, angiogenesis and vascular maturation [2, 8, 9]. Intracellularly, S1P regulates Ca^{2+} homeostasis and mobilization, promotes cell growth and suppresses apoptosis [2, 8, 10, 11].

mRNAs of the Sphk1 and the Sphk2 genes are expressed in rat pancreatic islets and INS-1 insulinoma cell lines and the activities of both of the SphK enzymes are present [12]. In addition, S1P receptor isoforms 1-4 were detected in rat and mouse islets and INS-1 cells [7]. In β -cells, cytokines including IL-1 β and TNF- α are major stress inducers implicated in the development of diabetes [13-17] and have been shown to activate SphK1 in INS-1 cells [12]. Ceramide is formed from S1P and sphingomyelin breakdown. Ceramide can be produced in response to inflammatory cytokines (e.g., TNF α or IL-1 β) or by excessive deposition of saturated fats, and inhibits insulin gene expression, blocks β -cell proliferation, and induces β -cell cytotoxicity and apoptosis [14, 18]. S1P can oppose ceramide actions including apoptosis [18], suggesting that the ceramide- S1P balance controls cellular responses [19]. In β -cells, S1P promotes growth and survival and augments glucose-stimulated insulin secretion [13, 14, 20, 21].

The present study explored the role of SphK1 on insulin secretion and synthesis in rat insulinoma INS-1 832/13 cells and showed that knockdown of Sphk1 gene expression resulted in a significant decrease in insulin synthesis and secretagogue-induced insulin secretion.

2.0 Materials and Methods

D-erythro-sphingosine was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Sphingosine 1-phosphate and N, N-dimethylsphingosine were from Cayman Chemical Company (Ann Arbor, Michigan). [γ - ^{32}P]ATP (3000 Ci/mmol) was from PerkinElmer (Boston, MA). Chemicals, in the highest purity available, were from Sigma Chemical (St. Louis, MO). pSilencerTM hygro and pSilencerTM puro were from Ambion (Austin, TX). The INS-1/832/13 cell line was a gift from Chris Newgard [22].

2.1 Generation of Sphk1 knockdown and control cell lines

The control CHS cell line was described previously [23]. The Sphk1 targeting vector contains 64- or 65-bp DNA inserts that code for shRNAs cloned into the BamHI and the HindIII sites of plasmid pSilencer 2.1-U6/hygro downstream of the U6 promoter. For stable expression of shRNAs that are processed in vitro to generate siRNA that targets Sphk1 mRNA, the vectors were transfected into INS-1 832/13 cells and the hygromycin resistant stable cell lines were isolated as described previously [23]. All targets were 19 nucleotides long. The rat Sphk1 mRNA (GenBankTM accession number NM_133386) targets used were SK11: CAGCTTCTTTGAACTACTA that corresponds to nucleotides 2042-2060; SK14: GCAGCTTCTTTGAACTACT, corresponds to nucleotides 2041-2059; and SK 2320: CTACAGGAAGGTAGGCCAG, corresponds to nucleotides 2320-2338 (Supplemental Table 1).

2.2 Real Time Quantitative Reverse Transcription PCR (qRT-PCR) mRNA quantification

Cells grown on 100-mm plates as described previously [23] were harvested at 60 – 80% confluence. The cells were washed twice with 10 ml of phosphate-buffered saline and then lysed and the total RNA was prepared using the RNeasy-minikit (Qiagen), with on-column deoxyribonuclease digestion (RNase-free DNase set, Qiagen). cDNA was prepared using 1.5 µg of total RNA using the RETROscript™ kit (Ambion/Applied Biosystems) with oligo(dT) primers. Real-time PCR was performed on a Bio-Rad MyiQ™ single color real-time PCR detection system. A standard curve prepared from CHS cDNA was included in each run for relative quantitation. Quantification of glutamate dehydrogenase mRNA was used as an internal control. Real time quantification of Sphk1 mRNA was carried out using the forward primer AGCATATGGACCTCGACTGC, and the reverse primer GCACAGCTTCACACACCATC. The primers for Glut1 (NM_012570) were forward CGAGAAGCAGTTGACCAAATCC and reverse CACTCCTCCAGCATTTCAGGTAGAG. The primers for Ins1 and Ins2 mRNA were: Ins1 forward ACCATCAGCAAGCAGGTCAT, and Ins1 reverse CACTTGTGGTCTCCTCACTT, Ins2 Forward CCTGCTCATCCTCTGGGAGCCCCGC and Ins2 reverse CTCCAGTGCCAAGGTCTGAAGGTCA. Real time quantification of Sphk2 mRNA was carried out using the forward and reverse primers described previously [24].

2.3 SphK enzyme assay

Cells were lysed by freeze/thawing in SphK buffer, containing 20 mM Tris buffer pH7.4, 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 20 mM sodium orthovanadate, 15 mM sodium fluoride, 0.5 mM deoxy pyridoxine and 40 mM β-glycerophosphate to inhibit S1P phosphohydrolase and lyase activities and protease inhibitor cocktail from Pierce. The lysate was centrifuged at 14000 × g for 20 min to generate a supernatant fraction that was used for measuring enzyme activities. SphK activity was determined using d-erythro-sphingosine and [³²P]ATP as substrates in the absence (basal activity) or the presence of either 0.4 M KCl or 1% Triton X-100. Radiolabeled S1P was quantitated following thin layer chromatography as described previously [12]. Radioactive spots were identified by autoradiography using X-Ray films and the radioactivity in the spots was quantified by liquid scintillation spectrometry. Specific activity is expressed as picomoles of S1P produced per minute per mg protein.

2.4 Insulin release experiments and total insulin measurements

Insulin release and total insulin measurements were described previously [23]. Briefly, insulin release was performed in 24-well tissue culture plates. One day before an insulin release experiment was to be performed, the glucose concentration in the tissue culture medium was reduced to 5 mM. Two hours before the experiment, the medium was replaced with Krebs-Ringer bicarbonate buffer, pH 7.3 (modified to contain 15 mM Hepes and 15 mM NaHCO₃ with the NaCl concentration adjusted to maintain osmolarity at 310) containing 3 mM glucose and 0.5% bovine serum albumin (BSA). Cells were washed once with the Krebs-Ringer Hepes BSA solution, and insulin release was studied in 1 ml of this same solution in the presence or absence of secretagogues. After 1 h at 37 °C, samples of incubation solution were collected and centrifuged to sediment any cells floating in the incubation solution. An aliquot of the supernatant fraction was removed and saved for insulin measurements by a standard radioimmunoassay as previously described [23]. The plates were then washed once with Krebs-Ringer solution containing no BSA, water was added to the plates, and the mixture containing the cells was removed and saved for estimation of total protein by the Bradford method using a dye reagent from Bio-Rad. To

measure total insulin content of cells, cells from individual wells incubated only in Krebs-Ringer Hepes BSA solution in absence of secretagogues were suspended in 1 ml of 75% ethanol, 1.5% HCl and 23.5% H₂O and used to determine the insulin content of the sample as previously described [23]. The mixture was appropriately diluted to estimate the insulin concentration by radioimmunoassay.

In certain instances CHS cells were grown in the presence or absence of 4 μ M DMS for 68 h. Subsequently, cells were plated in medium only or in medium containing 4 μ M DMS. After 48 h the medium was replaced with medium containing 5 mM glucose (as a control) or medium plus 5 mM glucose and 4 μ M DMS and insulin release and total insulin were measured 24 h later as described [23]. Alternatively, the total insulin content of CHS and INS-1 832/13 cells grown in the presence or absence of DMS was assessed after 72 h or 144 h. The cells were washed once with phosphate-buffered saline and treated with 0.05% trypsin, 0.5 mM EDTA in Hepes-buffered saline solution, followed by RPMI 1640 medium containing 10% fetal bovine serum. Cells were washed three times with phosphate-buffered saline and suspended in KMSH solution (220 mM mannitol, 70 mM sucrose, and 5 mM potassium Hepes buffer, pH 7.5) containing protease inhibitor mixture (Pierce). An aliquot was used for determination of total protein using Bradford method and a dye reagent from Bio-Rad. Another aliquot was mixed with 1 ml of 75% ethanol, 1.5% HCl and 23.5% H₂O and used to determine the insulin content of the sample as previously described [23].

2.5 Construction of Sphk1 rescue vector

The rat Sphk1 cDNA clone in pExpress1 and the human SPHK1 cDNA clone in pCMV-SPORT6 were purchased from Open Biosystems. The clones express rat SphK1 and human SPHK1 under the control of the CMV promoter. Using standard recombinant techniques, a puromycin gene cassette was inserted in the above vectors that were used subsequently to generate puromycin resistant stable cell lines that express either rat Sphk1 or human SPHK1 to study their effect on rescue of Sphk1 knockdown. The puromycin gene together with the SV40 promoter derived from pSilencer 2.1 U6 Puro (Ambion) was cloned into pExpress Sphk1. Both plasmids were digested with PvuI and the 2221 bp PvuI fragment containing the puromycin gene and SV40 promoter from pSilencer 2.1 U6 Puro was used to replace the 1044 bp PvuI fragment from pExpress Sphk1 and the 1202 bp PVUI fragment from pCMV-SPORT6 Sphk1. Plasmids designated pSK5 and pSK18h containing the puromycin gene and expressing rat Sphk1 and human SPHK1, respectively, were identified.

Plasmids pSK5 and pSK18h were used to transfect Sphk1 deficient and control cell lines. The transfected cells were selected in the presence of 150 ng/ml puromycin (832/13 SK) or 150 ng/ml puromycin and 100 μ g/ml of hygromycin. The resulting stable cell lines overexpressing rat Sphk1 were designated SK11-5, SK14-5 and CHS-5, 832/13 SK and the cell lines overexpressing human SPHK1 were designated SK11-18h and SK14-18h. To delete the SK1 gene from pSK5 DNA, a control vector was prepared by digestion of pSK5 DNA with BamHI and XhoI followed by mung bean nuclease treatment and religation. This operation removes the entire Sphk1 gene from pSK5 vector. The resulting plasmid was designated pSK-BX which was used to generate the control cell line CHS-20BX.

2.6 Measurement of control enzyme activities

The cells from a single 150 mm tissue culture plate were washed once with phosphate-buffered saline and treated with 0.05% trypsin, 0.5 mM EDTA in Hepes-buffered saline (PBS), followed by RPMI 1640 tissue culture medium containing 10% fetal bovine serum. Cells were washed three times with PBS and suspended in KMSH solution (220 mM mannitol, 70 mM sucrose, and 5 mM potassium Hepes buffer, pH 7.5) containing protease inhibitor mixture. The cell homogenate was obtained by two cycles of freezing at -80 °C and

thawing and the whole cell homogenate was centrifuged at $20,000 \times g$ for 10 min. The supernatant fraction was collected and used for enzyme assays using a SpectraMaxM2 spectrophotometer (Molecular Devices). The activities of cytosolic NADP-dependent malic enzyme1 (ME1), NADP isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase and ATP citrate lyase were measured as described [25, 26].

2.7 Immunohistochemistry

Immunohistochemistry was performed on cells grown on polylysine-coated slides, fixed with paraformaldehyde and blocked with normal goat serum for 1 hour. To stain for insulin, slides were incubated with guinea pig anti-insulin serum (Dako Corp.) (1:1000 dilution) at 7° overnight followed by Texas Red anti-guinea pig IgG (1:200 dilution) for 1 h at room temperature before viewing under a microscope [27].

3.0 Results

To evaluate the role of SphK1 in insulin release, we generated stable cell lines with knocked down Sphk1 activity using shRNA-expressing vectors that target the rat Sphk1 mRNA. To determine whether two cell lines with lowered Sphk1 mRNA, enzyme activity and glucose-stimulated insulin release could be rescued by overexpression of the SPHK1 gene, we generated cell lines that overexpress rat or human SPHK1 mRNA.

3.1 qRT- PCR

Quantitative RT-PCR analysis of mRNA in Sphk1 knockdown and control cell lines indicated that SK11 and SK14 cell lines showed a significant decrease in Sphk1 mRNA. Values were normalized to an internal standard (glutamate dehydrogenase mRNA). The level of Sphk1 mRNA in SK11 and SK14 cell lines was lowered by approximately 52% and 77%, respectively, as compared with the CHS cell line (Fig. 1). The Sphk1 mRNA in SK11 and SK 14 overexpressing exogenous rat Sphk1 was increased by 58-fold (SK11-5) and 20-fold (SK14-5) over the CHS control, respectively. These increases correspond to 119-fold and 89-fold over the corresponding knockdown cell lines, respectively. Figure 1 also suggests that the siRNA expressed in the SK14-5 cell lines is more effective at silencing Sphk1 mRNA than siRNA expressed in the SK11-5 lines based on the observation that Sphk1 mRNA expression is threefold lower in the SK14-5 line. In agreement with the qRT-PCR data, the SphK1 enzyme activity measured in the presence of Triton X-100 (to inhibit SphK2 and activate SphK1) showed significant activation of SphK1 activity in SK11-5 as compared with the SK14-5 cell line (Fig. 2 C). The lower Sphk1 mRNA and SphK1 enzyme activity in SK14-5 could be due to lowered expression of the exogenous Sphk1 gene in the SK14-5 cells.

3.2 SphK1 activity

Panels A, B and C of Figure 2 show SphK activities in the supernatant fractions of several Sphk1 knockdown and control cell lines including SK11, SK14, CHS, SK11-5, SK14-5, CHS-20BX and 832/13 measured by the formation of [32 P]S1P in the absence (basal activity) or presence of either KCl or Triton-X100. Triton X-100 was previously shown to activate SphK1 and inhibit SphK2, and KCl was found to inhibit SphK1 but not SphK2 [12]. The assay measures both SphK1 and SphK2 activities. In SK14-5 and SK11-5 knockdown cell lines that overexpressed the rat Sphk1, SphK1 activity was 4- and 15-fold higher than the activities in SK14 and SK11, respectively (Fig. 2A). In the presence of KCl to optimize SphK2 activity, extracts of SK14 and SK11 cells with suppressed Sphk1 expression and from untransfected 832/13 and CHS cells, [32 P]S1P formation was similar among the four extracts (Fig. 2B). With KCl present, [32 P]S1P production by the Sphk1 overexpressing

SK14-5 and SK11-5 cell lines was still 2.5- and 12-fold higher than in the SK14 and SK11 cells, respectively (Fig. 2B), suggesting that KCl did not completely inhibit SphK1 activity in these cell preparations. In the presence of Triton X-100, SphK activity was over 41-fold higher for SK11-5 and 16-fold higher for SK14-5 than in SK11 and SK14, respectively (Fig. 2C). Furthermore, in agreement with qRT-PCR data, the results indicate that the labeled S1P produced by the SK11-5 cell supernatant was over 10-fold higher than that from the SK14-5 cells. All the cell lines in the present study were derived from the INS-1 832/13 cell line and therefore should have similar SphK2 levels. As shown in Figure 3, in agreement with the activation of SphK in the presence of KCl, the level of SphK2 protein as determined by Western blotting was essentially unchanged for the control and Sphk1 knockdown cell lines (Fig. 3). In addition, Sphk1 knockdown as well as Sphk1 overexpression did not change the level of Sphk2 mRNA (data not shown.) indicating there was no compensation by the Sphk2 gene for silencing of Sphk1 mRNA. Furthermore, the extremely low SphK activities in the CHS, CHS-20BX, 832/13, SK11 and SK14 cell lines observed in the presence of Triton X-100 represent an underestimate of SphK1 activity (residual SphK2 activity and the presence of salts in the crude cytosolic fraction) and, therefore, the degree of Sphk1 silencing in SK11 and SK14 based on the standard assay for SphK1 enzyme activity is probably also underestimated.

3.3 Sphk1 knockdown does not alter the levels of control enzymes

The activity of NADP isocitrate dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase and ATP citrate lyase were measured as controls in the Sphk1 knockdown and control cell lines. Activities of these enzymes in the Sphk1-targeted cell lines were not significantly different from those in the control CHS cell line (Table 1).

3.4 Knockdown of Sphk1 lowered insulin release

Stable expression of siRNA that targeted Sphk1 mRNA inhibited glucose-stimulated insulin release by 50% to 90% in SK11, SK2320 and SK14 cell lines, as compared to the control CHS and INS-1 832/13 cell lines (Fig. 4A). Furthermore, insulin release induced by pyruvate, leucine, BCH or BCH plus glutamine was also inhibited in the SK14 cell line (Figure 4B). Similar results were obtained with the various siRNAs targeted to different sequences in the Sphk1 mRNA, suggesting that these effects were not due to off-target effects.

3.5 Total insulin content of Sphk1 knockdown cell lines

Knockdown of the Sphk1 gene resulted in a mild (29% for SK2320), to severe reduction (>60% for SK11 and SK14) of the total insulin contents (Fig. 5A). The decrease in insulin content was correlated with insulin gene *Ins1* mRNA levels using qRT-PCR, where it was observed that the SK11 and SK14 cell lines showed significant decreases in rat *Ins1* mRNA but not *Ins2* mRNA. The levels of *Ins1* mRNA were reduced by approximately 38% and 83% in SK11 and SK14 cell lines, respectively. Both cell lines contained levels of *Ins2* mRNA similar to the control cell lines (Fig. 5B). The results suggest that SphK1 plays a role in the *Ins1* gene transcription.

3.6 Inhibition of SphK by N,N-dimethylsphingosine (DMS) reduces insulin secretion and insulin content of CHS and INS-1 832/13 cell lines

To gain further insight on the role of SphK1 on insulin secretion and insulin synthesis, we investigated the effect of DMS, a competitive inhibitor of the SphK enzymes [28], on insulin secretion and insulin content using the CHS cell line. CHS was grown in the presence or

absence of DMS. Figure 6A shows that the addition of DMS to the culture medium lowered glucose-stimulated insulin release. Furthermore, DMS treatment also lowered the total insulin content of both CHS and INS-1 832/13 cell lines by over 60% (Fig. 6B and C). Under the same conditions, the addition of DMS did not affect the levels of NADP cytosolic isocitrate dehydrogenase measured as a control enzyme (Fig. 6C).

3.7 Sphk1 rescue

The overexpression of the rat Sphk1 enzyme activity in the SK11 cell line, but not the SK14 cell line (SK14-5) correlated with restored glucose-stimulated insulin secretion and insulin content relative to that of the control cell line CHS (Fig. 7A and C). In the SK14-5 cell line, the Sphk1 mRNA and enzyme activity was much higher than that of control cell lines (see Figures 1 and 2). Similar results were observed in cell lines SK11-18h and SK14-18h with overexpressed human SPHK1 (Fig. 7B and C). The SphK1 activity in the SK14-5 cell line relative to that of the SK11-5 cell line was 13.5%, 65% and 9% under basal activity and activity in the presence of KCl and TritonX-100, respectively (Fig. 2). Furthermore, the Sphk1 mRNA levels in SK14-5 cells was 3-fold lower than those in SK11-5 cells and 20-fold higher than the control cell lines (Fig. 1). The SK14 and SK11 cell targets differ in the first and last nucleotides of the Sphk1 mRNA target sequence (Table 1). Overexpression of rat Sphk1 in the control CHS cell line and the INS-1 832/13 parent cell line did not affect glucose stimulated insulin release and was associated with a 30% reduction of insulin content in CHS-5 as compared to the CHS cell line (Fig. 7A and C).

3.8 Immunohistochemistry

Insulin immunofluorescence staining of SK14, CHS and INS-1 832/13 cell lines showed that the insulin content in the SK14 cell line was severely decreased as compared to the CHS control cell line and the INS-1 832/13 parent cell line (Supplemental Figure 1). The level of insulin was 65% lower in the SK14 cell line compared to the control cell lines. The lower total insulin content in the SK14 cell line is consistent with the lower Ins1 mRNA levels as determined by qRT-PCR (Fig. 5B)

4.0 Discussion

A role for SphK1 in insulin-producing cells has not previously been reported. The transfection of INS-1 832/13 cells with shRNA targeting Sphk1 mRNA lowered the mRNA that encodes the enzyme by at least 50% or more. Two Sphk1 knockdown cell lines SK11 and SK14 showed impaired insulin release in response to stimulation with glucose and other secretagogues, suggesting that S1P produced by SphK1 plays a major role in mediating the secretory activity of the cells. Whether this response is a result of direct interaction of S1P with metabolic pathways or an effect(s) distal to metabolic stimulation that fuels secretion is not known. However, when Sphk1 was overexpressed in SK11 cells (SK11-5 cells), there was a 60-fold increase in Sphk1 mRNA levels compared with SK11 cells and a restoration of insulin release. The poor recovery of insulin release in the SK14-5 cell line may be related to the fact that the Sphk1 mRNA was not expressed in as high a level in the SK14-5 cell line as in the SK11-5 cell line. The protein level of SphK2 was unaffected by the Sphk1 mRNA suppression, suggesting that the expression levels of the two isozymes are not linked. A decrease in the rat Ins1 mRNA was also accompanied by a more than 50% reduction in the total insulin content of the cells. The loss of mRNA for the major of the two rat insulin genes expressed in INS-1 832/13 cells suggests that S1P mediates transcriptional activity or the stability of Ins1 mRNA levels in the cells. An inhibitor of SphK enzyme activity, DMS, had dramatic inhibitory effects on insulin release in the control CHS cell line and somewhat lesser but significant effects on insulin content during long-term culture

(hours to 3 days) of CHS and INS-1 832/13 cells. These results also suggest that S1P production in INS-1 cells is related to the normal function of the cells for secretion and insulin production. It is possible that S1P produced by SphK1 interacts with and modifies the activity of one or more transcription factors that regulate the Ins1 promoter [29, 30]. The reduced insulin content could be due to a lowering of glucose/insulin-dependent up-regulation of insulin gene transcription [31-35].

The effect of Sphk1 knockdown on insulin secretion could also be due to changes in the integrity of lipid rafts. Lipid rafts are cholesterol- and sphingolipid-rich plasma membrane domains that contain a variety of proteins. There is evidence suggesting that membrane lipid rafts act as regulators of exocytosis [36-38] and S1P induces the recruitment of cell membrane raft proteins [38]. SphK1 translocates to the plasma membrane lipid rafts, where its substrate, in a process dependent on the phosphorylation of the SphK1 protein, also increases SphK1 activity [39, 40]. The reduced insulin secretion in the Sphk1 knockdown cell lines could be due to changes in the lipid composition of the plasma membrane [40-47]. The importance of lipids as regulators of insulin content and insulin secretion in INS-1 832/13 cells is also indicated by lowered lipid synthesis, insulin content and insulin secretion that occurs in cell lines in which other enzymes directly involved in lipid synthesis, such as fatty acid synthase (MJM and NMH, unpublished data), or involved in the synthesis of lipid precursors, such as acetoacetyl-CoA synthase or succinyl-CoA:3-ketoacid-CoA transferase, have been knocked down with siRNA technology [26, 48].

5.0 Conclusions

The current study of shRNA mediated Sphk1 knockdown indicates that the activity of SphK1 is important for the regulation of insulin synthesis and secretion. Knockdown of Sphk1 mRNA and inhibition of SphK activity reduced secretagogue-induced insulin secretion and total insulin content, whereas overexpression of Sphk1 mRNA increased SphK1 enzyme activity and restored insulin biosynthesis and insulin release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DMS	N,N-Dimethylsphingosine
S1P	Sphingosine 1 phosphate
SphK	Sphingosine kinase
S1PR1-5	S1P receptors 1- 5

References

1. Olivera A, Kohama T, Edsall L, Nava V, Cuvillier O, Poulton S, Spiegel S. Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J. Cell Biol.* 1999; 147:545-558. [PubMed: 10545499]

2. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol.* 2003; 4:397–407. [PubMed: 12728273]
3. Jarman KE, Moretti PA, Zebol JR, Pitson SM. Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1. *J. Biol. Chem.* 2010; 285:483–492. [PubMed: 19854831]
4. Johnson KR, Becker KP, Facchinetti MM, Hannun YA, Obeid LM. PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane: Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (Pma). *J. Biol. Chem.* 2002; 277:35257–35262. [PubMed: 12124383]
5. Pitson SM, Xia P, Leclercq TM, Moretti PA, Zebol JR, Lynn HE, Wattenberg BW, Vadas MA. Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signaling. *J. Exp. Med.* 2005; 201:49–54. [PubMed: 15623571]
6. Pyne S, Pyne NJ. Sphingosine-1-phosphate signaling in mammalian cells. *Biochem. J.* 2000; 349:385–402. [PubMed: 10880336]
7. Laychock SG, Tian Y, Sessanna S. Endothelial differentiation gene (EDG) receptors in pancreatic islets and INS-1 cells. *Diabetes.* 2003; 52:1986–1993. [PubMed: 12882914]
8. Saba JD, Hla T. Point-counterpoint of sphingosine 1-phosphate metabolism. *Circ. Res.* 2004; 94:724–734. [PubMed: 15059942]
9. Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science.* 2002; 296:346–349. [PubMed: 11923495]
10. Olivera A, Spiegel S. Sphingosine kinase: a mediator of vital cellular functions. *Prostaglandins.* 2001; 64:123–134. [PubMed: 11331100]
11. Young KW, Nahorski SR. Sphingosine 1-phosphate: a Ca^{2+} release mediator in the balance. *Cell Calcium.* 2002; 32:335–341. [PubMed: 12543093]
12. Mastrandrea LD, Sessanna SM, Laychock SG. Sphingosine kinase activity and sphingosine-1 phosphate production in rat pancreatic islets and INS-1 cells, response to cytokines. *Diabetes.* 2005; 54:1429–1436. [PubMed: 15855330]
13. Mandrup-Poulsen T, Bendtzen K, Dinarello CA, Nerup J. Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic β -cell cytotoxicity. *J. Immunol.* 1987; 139:4077–4082. [PubMed: 3320203]
14. Laychock SG, Sessanna SM, Lin MH, Mastrandrea LD. Sphingosine 1-phosphate affects cytokine-induced apoptosis in rat pancreatic islet β -cells. *Endocrinology.* 2006; 147:4705–4712. [PubMed: 16794003]
15. Cardozo AK, Heimberg H, Heremans Y, Leeman R, Kutlu B, Kruhøffer M, Ørntoft T, Eizirik DL. TTTA comprehensive analysis of cytokine-induced and nuclear factor-dependent genes in primary rat pancreatic β -cells. *J. Biol. Chem.* 2001; 276:48879–48886. [PubMed: 11687580]
16. Hoorens A, Stange G, Pavlovic D, Pipeleers D. Distinction between interleukin-1–induced necrosis and apoptosis of islet cells. *Diabetes.* 2001; 50:551–557. [PubMed: 11246874]
17. Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS. Nuclear factor B protects pancreatic β -cell from tumor necrosis factor- α –mediated apoptosis. *Diabetes.* 2003; 52:1169–1175. [PubMed: 12716748]
18. Taha TA, Mullen TD, Obeid LM. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochim. Biophys. Acta.* 2006; 1758:2027–2036. [PubMed: 17161984]
19. Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. Sphingosine kinases, sphingosine-1-phosphate, apoptosis and diseases. *Biochim. Biophys. Acta.* 2006; 1758:2016–2026. [PubMed: 16996023]
20. Shimizu H, Okajima F, Kimura T, Ohtani K, Tsuchiya T, Takahashi H, Kuwabara A, Tomura H, Sato K, Mori M. Sphingosine 1-phosphate stimulates insulin secretion in HIT-T 15 cells and mouse islets. *Endocr. J.* 2000; 47:261–269. [PubMed: 11036869]

21. Fu F, Hu S, Li S, DeLeo J, Hoover J, Wang S, Lake P, Shi V. FTY720, a novel immunosuppressive agent with insulinotropic activity, prolongs graft survival in a mouse islet transplantation model. *Transplant Proc.* 2001; 33:672–673. [PubMed: 11267010]
22. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1 derived cells lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes.* 2000; 49:424–430. [PubMed: 10868964]
23. Hasan NM, Longacre MJ, Stoker SW, Boonsaen T, Jitrapakdee S, Kendrick MA, Wallace JC, MacDonald MJ. Impaired anaplerosis and insulin secretion in insulinoma cells caused by siRNA-mediated suppression of pyruvate carboxylase. *J. Biol. Chem.* 2008; 283:28048–28059. [PubMed: 18697738]
24. Mastrandrea LD, Sessanna SM, Del Toro A, Laychock SG. ATP-independent glucose stimulation of sphingosine kinase in rat pancreatic islets. *J. Lipid Res.* 2010; 51:2171–2180. [PubMed: 20371493]
25. Brown LJ, Longacre MJ, Hasan NM, Kendrick MA, Stoker SW, MacDonald MJ. Chronic reduction of the cytosolic or NAD(P)-mitochondrial malic enzyme does not affect insulin secretion in a rat insulinoma cell line. *J. Biol. Chem.* 2009; 284:35359–35367. [PubMed: 19858194]
26. MacDonald MJ, Smith AD III, Hasan NM, Sabat G, Fahien LA. Feasibility of pathways for transfer of acyl groups from mitochondria to the cytosol to form short chain acyl-CoAs in the pancreatic beta cell. *J. Biol. Chem.* 282:30596–30606. [PubMed: 17724028]
27. Hasan NM, Kendrick MA, Druckenbrod NR, Huelsmeyer MK, Warner TF, MacDonald MJ. Genetic association of the neuropilin-1 gene with type1 diabetes in children: Neuropilin-1 expression in pancreatic islets. *Diab. Res. Clin. Pract.* 2010; 87:e29–e32.
28. Edsall LC, Van Brooklyn JR, Cuviller O, Kleuser B, Spiegel S. N,N Diethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: Modulation of cellular levels of sphingosine 1-phosphate and ceramide. *Biochemistry.* 1998; 37:12892–12898. [PubMed: 9737868]
29. Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S, Spiegel S. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science.* 2009; 325:1254–1257. [PubMed: 19729656]
30. Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T, Sheldon Milstien S, Spiegel S. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature.* 2010; 465:1084–1058. [PubMed: 20577214]
31. Reddy D, Pollock AS, Clark SA, Sooy K, Vasavada RC, Stewart AF, Honeyman T, Christakos S. Transfection and overexpression of the calcium binding protein calbindin-D28k results in a stimulatory effect on insulin synthesis in a rat b cell line (RIN 1046-38). *Proc. Natl. Acad. Sci.* 1997; 94:1961–1966. [PubMed: 9050887]
32. Leibiger IB, Leibiger B, Berggren PO. Insulin feedback action on pancreatic L-cell function. *FEBS Letters.* 2002; 532:1–6. [PubMed: 12459452]
33. Macfarlane WM, Shepherd RM, Cosgrove KE, James RF, Dunne MJ, Docherty K. Glucose modulation of insulin mRNA levels is dependent on transcription factor PDX-1 and occurs independently of changes in intracellular Ca²⁺. *Diabetes.* 2000; 49:418–423. [PubMed: 10868963]
34. Lawrence MC, Bhatt HS, Watterson JM, Easom RA. Regulation of insulin gene transcription by a Ca²⁺-responsive pathway involving calcineurin and NFAT. *Mol. Endocrinol.* 2001; 15:1758–1767. [PubMed: 11579208]
35. Lawrence MC, Bhatt HS, Easom RA. NFAT regulates insulin gene promoter activity in response to synergistic pathways induced by glucose and glucagon-like peptide-1. *Diabetes.* 2002; 51:691–698. [PubMed: 11872668]
36. Salaun C, Gould GW, Chamberlain LH. Lipid raft association of SNARE proteins regulate exocytosis in PC12 cells. *J. Biol. Chem.* 2005; 280:19449–19453. [PubMed: 15769746]
37. Chamberlain LH, Burgoyne RD, Gould GW. SNARE proteins are highly enriched in lipid rafts in PC12 cells: implications for the spatial control of exocytosis. *Proc. Natl. Acad. Sci. USA.* 2001; 98:5619–5624. [PubMed: 11331757]
38. Salaun C, James DJ, Chamberlain LH. Lipid rafts and the regulation of exocytosis. *Traffic.* 2004; 5:255–264. [PubMed: 15030567]

39. Zhao J, Patrick A, Singleton PA, Brown ME, Steven M, Dudek SM, Garcia JGN. Phosphotyrosine protein dynamics in cell membrane rafts of sphingosine-1-phosphate-stimulated human endothelium: Role in barrier enhancement. *Cell. Signal.* 2009; 21:1945–1960. [PubMed: 19755153]
40. Hengst JA, Francy-Guilford JM, Fox TE, Wang X, Conroy EJ, Jong K, Yun JK. Sphingosine kinase 1 localized to the plasma membrane lipid raft microdomain overcomes serum deprivation induced growth inhibition. *Arch. Biochem. Biophys.* 2009; 492:62–73. [PubMed: 19782042]
41. Takahashi N, Kishimoto T, Nemoto T, Kadowaki T, Kasai H. Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. *Science.* 2002; 297:1349. [PubMed: 12193788]
42. van Meer G, Hein Sprong H. Membrane lipids and vesicular traffic. *Curr. Opin. Cell Biol.* 2004; 16:373–378. [PubMed: 15261669]
43. Deebaa F, Tahseena NH, Sharada KS, Ahmad N, Akhtara S, Saleemuddin M, Mohammad O. Phospholipid diversity: Correlation with membrane–membrane fusion events. *Biochim. Biophys. Acta.* 2005; 1669:170–181. [PubMed: 15893520]
44. Rogasevskaja T, Coorssen JR. Sphingomyelin-enriched microdomains define the efficiency of native Ca^{2+} -triggered membrane fusion. *J. Cell Science.* 2006; 119:2688–2694. [PubMed: 16757517]
45. Churchward MA, Rogasevskaja T, Brandman DM, Khosravani H, Nava P, Atkinson JK, Coorssen JR. Specific lipids supply critical negative spontaneous curvature - An essential component of native Ca^{2+} -triggered membrane fusion. *Biophys. J.* 2008; 94:3976–3986. [PubMed: 18227127]
46. Eliasson L, Abdulkader F, Braun M, Galvanovskis J, Michael B, Hoppa MB, Rorsman P. Novel aspects of the molecular mechanisms controlling insulin secretion. *J. Physiol.* 2008; 586:3313–3324. [PubMed: 18511483]
47. Xia F, Xie L, Mihic A, Gao X, Chen Y, Gaisano HY, Robert G, Tsushima RJ. Inhibition of cholesterol biosynthesis impairs insulin secretion and voltage-gated calcium channel function in pancreatic β -cells. *Endocrinology.* 2008; 149:5136–5145. [PubMed: 18599549]
48. Hasan NM, Longacre MJ, Seed Ahmed M, Kendrick MA, Gu H, Ostenson CG, Fukao T, MacDonald MJ. Lower succinyl-CoA:3-ketoacid-CoA transferase (SCOT) and ATP citrate lyase in pancreatic islets of a rat model of type 2 diabetes: knockdown of SCOT inhibits insulin release in rat insulinoma cells. *Arch. Biochem. Biophys.* 2010; 499:62–68. [PubMed: 20460097]

Highlights

shRNA knockdown studies were performed in the INS-1 832/13 cell line. Sphingosine kinase 1 gene (Sphk1) knockdown lowered insulin release. Insulin gene transcription and insulin contents were decreased in knockdown cells.

Overexpression of Sphk1 rescued insulin secretion in some knockdown cell lines.

Figure 1. Knockdown of Sphk1 expression in INS-1 832/13 cells by shRNA and overexpression of Sphk1 in the knockdown cell lines

INS-1 832/13 cells were transfected with shRNA-expressing plasmids and the levels of Sphk1 mRNA in the stable knockdown cell lines were measured by qRT-PCR. The results represent the means $\pm$ SE of three independent experiments (a, $P < 0.05$ vs. CHS Control; b, $P < 0.02$ vs. CHS control; c, $P < 0.01$ vs. CHS control; and d, $P < 0.001$ vs. CHS control).

Figure 2. Sphingosine kinase 1 enzyme activity in Sphk1 knockdown cell lines and the cell lines overexpressing the rat Sphk1

A representative autoradiogram of S1P produced, extracted, and isolated by TLC. Cytosolic SphK1 activity was determined in the presence of sphingosine and [32 P]ATP as described. The radioactivity in the spots corresponding to S1P was determined. Panel A shows the autoradiogram of SphK reaction under basal conditions. Panel B shows the autoradiogram of the SphK reaction in the presence of 0.4 M KCl and Panel C shows the autoradiogram of the SphK reaction in the presence of 1% Triton X-100. The numbers above the spots indicate the specific activity of SphK enzymes expressed as pmoles/min/mg protein.

Figure 3. Stable knockdown of Sphk1 mRNA expression does not affect the level of SphK2 protein

Western blot analysis of whole cell homogenate protein did not affect the levels of SphK2 protein in Sphk1 knockdown lines. The values below the lanes indicate the relative density of the SphK2 band relative to that of the CHS cell line (100%).

Figure 4. Stable knockdown of the Sphk1 mRNA by shRNA inhibits insulin secretion in INS-1 832/13 cells

Panel **A**, insulin released during a 1-h incubation of Sphk1 knockdown and control cell lines without or with 11.1 mM glucose. Panel **B**, insulin release of SK14 knockdown and CHS cell lines with 10 mM BCH, 10 mM leucine, 5mM pyruvate or 10 mM glutamine plus 10 mM BCH. Results are the means $\pm$ SE of 4-10 independent experiments with each experiments run in quadruplicate. (d, $P < 0.001$ vs. CHS control; e, $P > 0.05$ vs. CHS control)

Figure 5. Stable knockdown of the SphK1 mRNA reduces the Ins1 mRNA and the total insulin content

Panel **A**, shows the reduced insulin contents of Sphk1 knockdown cell lines relative to the CHS control. Panel **B**, shows that the relative Ins1 mRNA level was significantly reduced in the Sphk1 knockdown cell lines determined by qRT-PCR, using a CHS cDNA pool to prepare the standard curve. The level of Ins2 mRNA was not affected by Sphk1 knockdown. Values were normalized to an internal glutamate dehydrogenase mRNA. Results are the means $\pm$ SE of 3 experiments (d, $P < 0.001$ vs. CHS control; c, $P < 0.01$ vs. CHS control; and e, $P > 0.05$).

Figure 6. DMS, an inhibitor of SphK, lowers insulin release and insulin content in CHS cells
 Panels **A** and **B**, show glucose stimulated insulin release and the total insulin content of DMS-treated and untreated CHS cells, respectively. Panel **C**, shows the total insulin content of CHS and INS-1 832/13 cell lines grown in a medium containing various concentrations of DMS for 72 hours or 144 hours. Panel **D**, shows the level of NADP isocitrate dehydrogenase (ICDH) as a control in the cell preparations of the control cell lines described in panel **C**. Results are the mean $\pm$ SE of 4-7 independent cell preparations (Panels **A**, **B**, and **C**). (a, $P < 0.05$ vs. CHS Control; c, $P < 0.02$ vs. control; d, $P < 0.001$ vs. control; and e, $P > 0.05$).

Figure 7. Effect of overexpression of exogenous Sphk1 in Sphk1 knockdown cells on insulin release, total insulin content and Sphk1 mRNA

Overexpression of either rat (Panel A) or human (Panel B) SPHK1 in the presence of Sphk1 knockdown restored glucose stimulated insulin secretion in the SK11 but not the SK14 knockdown cells. Panel C shows that overexpression of either rat or human SPHK1 in Sphk1 knockdown restores the total insulin content in the SK11 cells but not SK14 cells. Results are the mean $\pm$ SE of 4-10 experiments with each experiment run in quadruplicate. (c, $P < 0.01$ vs. CHS control; d, $P < 0.001$ vs. CHS control; and e, $P > 0.05$ vs. CHS control).

Table 1
Control enzyme activities in the SphK1 deficient cell lines

Enzyme rates are expressed as a percentage of the control CHS cell line equal to 100%. Average specific enzyme activities defined as 100% in nmol of product formed/min/mg of protein were 99 for NADP isocitrate dehydrogenase (ICDH), 46 for cytosolic malic enzyme (ME1), 110 for glucose-6-phosphate dehydrogenase (G6PD), and 172 for ATP citrate lyase (ACL). Results are the mean $\pm$ SE from 4 independent cell preparations for ME1, 3 for NADP isocitrate dehydrogenase (ICDH) and 2 for G6PD and ATP citrate lyase.

Cell lines	ICDH % of Control	ME1 % of Control	G6PD % of Control	ACL % of Control
CHS	100	100	100	100
832/13	106.4 $\pm$ 6.9 ^a	ND	91.9 $\pm$ 1.4 ^a	121.94 $\pm$ 11.8 ^a
SK 11	82.5 $\pm$ 27 ^a	93.7 $\pm$ 2.3 ^a	82.1 $\pm$ 1.6 ^a	98.8 $\pm$ 6.6 ^a
SK 14	61.5 $\pm$ 17.8 ^a	79.2 $\pm$ 5.3 ^a	85.7 $\pm$ 1.7 ^a	71.2 $\pm$ 6.4 ^a
SK 2320	97.2 $\pm$ 13 ^a	102 $\pm$ 2.5 ^a	76.2 $\pm$ 1.7 ^b	88.2 $\pm$ 5.2 ^a

^aNo significant difference

^bP < 0.05 vs. CHS control.