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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.

Introduction

Sphingosine kinase isoforms 1 and 2 (SphK1[2] and SphK2) are lipid kinases that catalyze phosphorylation of sphingosine to generate sphingosine 1-phosphate (S1P) [1,2]. Activation 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 1–5 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-1β 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., TNFα or IL-1β) 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.

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[2] *Abbreviations used:* DMS, N,N-dimethylsphingosine; S1P, sphingosine 1 phosphate; SphK, sphingosine kinase; S1PR1–5, S1P receptors 1–5.

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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 Perkin–Elmer (Boston, MA). Chemicals, in the highest purity available, were from Sigma Chemical (St. Louis, MO). pSilencer™ hygro and pSilencer™ puro were from Ambion (Austin, TX). The INS-1/832/13 cell line was a gift from Chris Newgard [22].

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 (GenBank™ accession number NM_133386) targets used were SK11: CAGCTTCTTTGAACTACTA that corresponds to nucleotides 2042–2060; SK14: GCAGCTTCTT TGAACTACT, corresponds to nucleotides 2041–2059; and SK 2320: CTACAGGAAGGTAGGCCAG, corresponds to nucleotides 2320–2338 (Supplemental Table 1).

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 Glud1 (NM_012570) were forward CGAGAAGCAGTTGACCAAATCC and reverse CACTCCTCCAGCATTCAGGTAGAG. The primers for INS1 and INS2 mRNA were: INS1 forward ACCATCAGCAAGCAGGTCAT, and INS1 reverse CACTTGTGGGTCCTCCACTT, INS2 Forward CCTGCTCATCCTCTGGGAGCCCCGC and INS2 reverse CTCCAGTGCC AAGGTCTGAAGGTCA. Real time quantification of Sphk2 mRNA was carried out using the forward and reverse primers described previously [24].

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 deoxypyridoxine and 40 mM β-glycerophosphate to inhibit S1P phosphohydrolase and lyase activities and protease inhibitor cocktail from Pierce. The lysate was centrifuged at 14,000*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 [^{32}Pγ]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 min per mg protein.

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 $NaHCO_3$ 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% H_2O 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 μM DMS for 68 h. Subsequently, cells were plated in medium only or in medium containing 4 μM 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 μM 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 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 the 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% H_2O and used to determine the insulin content of the sample as previously described [23].

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