ATP-independent glucose stimulation of sphingosine kinase in rat pancreatic islets

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Abstract **Sphingosine kinase (SPHK) catalyzes sphingosine 1-phosphate production, promoting cell survival and reducing apoptosis in isolated rat pancreatic islets. Glucose, the primary islet β-cell growth factor and insulin secretagogue, increased islet SPHK activity by 3- to 5-fold following acute (1 h) or prolonged (7 days) stimulation. Prolonged stimulation of islets with glucose induced SPHK1a and SPHK2 mRNA levels; there were no changes in SPHK protein expression. To isolate the metabolic effects of glucose on SPHK activation, islets were stimulated with glucose analogs or metabolites. 2-deoxy-D-glucose (2-DG), an analog phosphorylated by glucokinase but not an effective energy source, activated SPHK similarly to glucose. In contrast, 3-o-methylglucose (3-oMeG), which is transported but neither phosphorylated nor metabolized, did not increase islet SPHK activity. Glyceraldehyde and α-ketoisocaproic acid (KIC), metabolites that stimulate glycolysis and the citric acid cycle, respectively, did not activate islet SPHK. Moreover, inorganic phosphate blocked glucose-induced SPHK activation. A role for SPHK activity in β-cell growth was confirmed when small interfering (si)SPHK2 RNA transfection reduced rat insulinoma INS-1e cell SPHK levels and activity and cell growth. Glucose induced an early and sustained increase in islet SPHK activity that was dependent on glucose phosphorylation, but independent of ATP generation or new protein biosynthesis. Glucose-supported β-cell growth appears to be in part mediated by SPHK activity.—** Mastrandrea, L. D., S. M. Sessanna, A. Del Toro, and S. G. Laychock. **ATP-independent glucose stimulation of sphingosine kinase in rat pancreatic islets. *J Lipid Res*. 2010.** 51: **2171–2180.**

Supplementary key words SPHK • islets of Langerhans • sphingosine-1-phosphate

Sphingosine kinases (SPHK) catalyze the metabolism of sphingosine to the bioactive sphingolipid sphingosine 1-phosphate (S1P) (1). S1P is a pleiotropic signaling molecule with actions both on a family of G-protein-coupled receptors in the plasma membrane and largely undefined intracellular actions (2). S1P receptors (S1PR) activate downstream signaling pathways that modulate cell differentiation, growth and proliferation, inflammation, and apoptotic pathways, among other things (3–6). In the pancreatic islet of Langerhans, S1P has been reported to inhibit adenylyl cyclase activation and insulin secretion (7) and to protect islet cells from cytokine induced apoptosis (8).

Two isoforms of SPHK, SPHK1a and SPHK2, have been identified. The stimulation of SPHK1 with resulting S1P biosynthesis has been reported to play a role in Ca^{2+} mobilization, cell survival, cell motility, gene activation and DNA synthesis, endothelial nitric oxide synthase activity, phosphorylation of Akt and ERK1/2, cell growth, differentiation and proliferation, and vesicular trafficking, among other things (2). In contrast, SPHK2 reportedly augments apoptosis and inhibits DNA biosynthesis and cell growth in some cell types (9, 10). SPHK has been reported to be activated by numerous stimuli, including cytokines, phorbol myristic acetate, growth factors, hormones, cyclic AMP, glucose, and ATP (2). Recently, we reported that SPHK activity is present in pancreatic islets and INS-1e rat insulinoma cells and that this activity increases in response to cytokines implicated in the development of type 1 diabetes mellitus (11).

Glucose is the primary regulator of pancreatic islet β-cell insulin secretion and growth. Glucose metabolism in β-cells stimulates insulin secretion, activates signal transduction pathways (12), and modulates gene transcription (13) and β-cell growth and survival (14). Glucose stimulatory responses are dependent upon facilitated glucose transport into the β-cell, followed by phosphorylation of glucose by glucokinase, which catalyzes the rate-limiting step in glucose metabolism. Glucose-6-phosphate metabolism through glycolysis and the mitochondrial citric acid

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Abbreviations: 2-DG, 2-deoxy-D-glucose; 3-oMeG, 3-o-methylglucose; KIC, α-ketoisocaproic acid; SPHK, sphingosine kinase; S1P, sphingosine 1-phosphate; S1PR, sphingosine 1-phosphate receptor.

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cycle generates ATP. In addition to participating in many metabolic actions, ATP binds to and inhibits ATP-sensitive K^+ channels, leading to β-cell depolarization, Ca^{2+} influx, and insulin secretion (15). In contrast to the acute stimulatory effects of glucose on β-cells, chronic glucose stimulation in vitro (16) and in vivo (17) leads to β-cell dysfunction with deterioration of insulin secretion, impairment in insulin production (18), and downregulation of signal transduction pathways (19). Ultimately, glucose-induced destruction of the islet via apoptosis can occur (20, 21).

As glucose stimulation of islet β-cells contributes to changes in insulin biosynthesis and secretion as well as growth and mitogenesis (22), the potential for mediation of such effects by SPHK is a focus of this study. A role for glucose in β-cell SPHK expression and activity in pancreatic islet cells was determined.

MATERIALS AND METHODS

Materials

Sphingosine was from Biomol Research Laboratory (Plymouth Meeting, PA). [γ^{32}P]ATP (3,000 Ci/mmol) was from Amersham (Piscataway, NJ). FBS was from Atlanta Biologicals (Norcross, GA). Culture media and molecular biology reagents were from Invitrogen (Grand Island, NY). QuantumRNA Classic 18S standards (488 bp) were from Ambion (Austin, TX). Essentially fatty acid free, fraction V, BSA (FAF-BSA), ATP, glucose analogs, and other reagents were from Sigma Chemical (St. Louis, MO). Collagenase was obtained from Crescent Chemical Co. (Islandia, NY). Protein assay reagent and iQ SYBR Green Supermix were from Bio-Rad Laboratories (Hercules, CA). SPHK1 and SPHK2 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Tissue preparation and cell culture

Rat pancreatic islets were isolated and incubated essentially as described previously (23). Animal procedures were approved by the Institutional Animal Care and Use Committee (University at Buffalo). Islets were cultured in CMRL-1066 medium, as described previously (8). For long-term (1–7 days) glucose treatment, islets were cultured in CMRL-1066 containing 5.5 mM (basal) or 11 mM (high) glucose, with media changes every other day. For 1-h treatment, isolated islets were washed and incubated in oxygenated Krebs Ringer bicarbonate HEPES (KRBH) buffer (pH 7.4) containing 0.01% insulin-free FAF-BSA, HEPES (16 mM), and glucose or glucose analogs as indicated. The rat insulinoma cell line, INS-1e (generous gift of Dr. Claes Wollheim, Geneva, Switzerland) were cultured in standard RPMI-1640 (glucose 11 mM) containing 10% FBS (24). For long-term glucose treatment, cells were first cultured in media supplemented with 5 mM glucose for 2 weeks to upregulate glucose-sensitive signaling. Cells were then treated with media containing increasing concentrations of glucose (5–17 mM) for 4 days. To determine stability of SPHK1 and SPHK2 protein, INS-1e cells were cultured in standard media. When cells reached 80% confluence, the cells were treated for 1–24 h with cycloheximide (10 μM), and protein levels were determined by Western blot.

mRNA quantitation

Islets were cultured in 5 mM or 11 mM glucose for 7 days with or without cytosine arabinoside (Ara-C; 10 μM) to limit the effect that cell proliferation might have on SPHK mRNA levels. Levels of SPHK1 and SPHK2 mRNA were determined by real-time QPCR (25). Islet RNA (15–20 islets per extract) was extracted following treatment as described previously (7). QPCR was carried out using primers to SPHK1a: sense, 5′-AGCATATGGACCTCGACTGC-3′; and antisense, 5′-GCACAGCTTCACACACCATC-3′ (122 bp), and to SPHK2: sense, 5′-CATCCTCTGGACCTGCTCTC-3′; and antisense, 5′-CACTGCACCCAGTGTGAATC-3′ (150 bp). QPCR was carried out with the iQ SYBR Green Supermix using a real-time PCR thermal cycler (Bio-Rad) according to the manufacturer's instructions. Reaction conditions were 95°C for 3 min initial denaturation, followed by 42 cycles of 95°C for 10 s and then 58°C for 30 s The reaction was followed by melt curve analysis to verify specificity. In each reaction, gene expression levels were normalized to the level of 18S rRNA and calculated using the $2^{-\Delta\Delta CT}$ method.

Assay of SPHK activity

Islets and cells were treated with glucose or glucose analogs for indicated times. Islets and harvested cells were lysed by freeze-thawing in SPHK buffer containing sodium orthovanadate (20 mM), sodium fluoride (15 mM), and β-glycerophosphate (40 mM) to inhibit S1P phosphohydrolase and lyase activities. Islet samples were sonicated briefly. SPHK activity was determined in islet or INS-1e cell cytosolic (14,000 *g*) fractions (30–60 μg protein) with the addition of sphingosine (50 μM) and [γ^{32}P]ATP (2.5 μCi, 1 mM), as described previously (11). Reactions were incubated for 60 min at 37°C with gentle shaking. [^{32}P]S1P was extracted and isolated by thin-layer chromatography and quantitated by phosphoimage densitometric analysis using Multi-Analyst software (Bio-Rad). Sample densities were converted to counts per minute (cpm). Protein concentrations of the islet lysates were determined using Bio-Rad protein assay. SPHK-specific activity is expressed as picomoles of S1P formed per min per mg of total protein. Average dpm recovered from a typical assay was 500 (islets) and 2000 (INS-1e cells).

Immunoblot analysis

INS-1e cells and islets were harvested in total protein lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM EDTA, pH 7.4, 1 mM PMSF, 10 μg/ml aprotinin, and 1% Triton X-100) and boiled. Equal amounts of whole-cell lysates per lane (50–60 μg, INS-1e cells; 25 μg, islets) were resolved by SDS-PAGE (12.5% gel) and transferred to PVDF membrane. Membranes were blocked using Tris-buffered saline with Tween 20 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.05% Tween 20; TBST) containing 5% powdered milk for 1 h, and then incubated 2 h at room temperature with the indicated SPHK antibodies (1:200 dilution). After washing three times with TBST, the membranes were probed with donkey α-goat horseradish peroxidase-conjugated secondary antibody to detect bands via WestPico chemiluminescence reagent (Pierce Biotechnology, Rockford, IL). Chemiluminescence signal was digitized using Fuji LAS-1000 Plus imager, and the band density was determined by densitometric scanning using Quantity-One Molecular Analyst software (Bio-Rad). For islet samples where total protein loaded was 25 μg, SPHK isoform expression was normalized to β-actin expression; SPHK isoform expression in INS-1e cells extracts was expressed as relative densitometric units. For INS-1e cell extracts, estimated half-life of SPHK isoforms was calculated using the equation natural $\log(C/C_0) = -kt$, where $C_0 = 100\%$, C = % expression at time = t, and k is the decay constant.

Generation of small interfering SPHK2 RNA constructs

SPHK2 knockdown was performed using sequence specific oligonucleotides that form short siRNA hairpin transcripts against SPHK2 in vivo (26). The target sequence for rat SPHK2 (Gen-

Bank accession number XM574450) corresponded to nucleotides 602-624. A target in the luciferase gene (siLuc) was used as a nontarget siRNA negative control (27). For each target, sense and antisense oligonucleotides were designed and synthesized as described (jura.wi.mit.edu/siRNAext/) (28). Oligonucleotides were annealed in buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA, and 50 mM NaCl. Following annealing, the oligonucleotide pair was ligated into Bgl II/Hind III linearized pSUPER. neo+gfp vector (26). siSPHK2-pSuper and siLuc-pSuper clones were confirmed by DNA sequencing.

Cell growth assay

INS-1e cells were transfected with pSuper vectors containing either siLuc or siSPHK2 insert using lipofectamine. Three days after transfection, cells were sorted by cytometry gating for GFP fluorescence: negative sort refers to GFP-negative cells and positive sort refers to GFP-containing cells. Sorted cells were plated at a density of 0.05×10^6 cells/ml and cultured in modified RPMI-1640 with 11 mM glucose and 5% FBS (24). INS-1 cell growth was determined by DNA quantitation. Cell DNA was quantitated at time zero, 5 days, and 11 days of cell culture. DNA was quantitated using fluorometric analysis, essentially as described (29). Only attached cells were quantitated for DNA as there was no discernable quantitative difference between the few floating cells among the treatment groups.

Statistical analysis

Values are means ± SE. Significant differences between treatment groups were determined by Student's *t*-test (paired) or one-way ANOVA with posthoc analysis using Student-Newman-Keuls multiple comparison test. QPCR results were analyzed by two-tailed, one-sample *t*-test. Values of $P \leqslant 0.05$ were accepted as significant.

RESULTS

Effect of short-term glucose stimulation on SPHK activity

We previously reported that β-cells show predominantly SPHK2 activity and little or no SPHK1 activity (11). The present study reports data for total SPHK activity in islets. To determine the effect of short-term glucose stimulation on β-cell SPHK activity, isolated islets were cultured for 1 h or 24 h at a physiological glucose concentration (basal, 5.5 mM) or with a hyperglycemic equivalent of 11 mM glucose. Total SPHK activity was quantitated as the phosphorylation of sphingosine to S1P in islet cytosolic fractions. Within 1 h of intact islet exposure to glucose (11 mM) stimulation there was a 2-fold increase in total SPHK activity (**Fig.1A**). SPHK activity remained elevated during islet glucose stimulation for 24 h with activity approximately 3-fold higher than basal values (Fig. 1A), and not significantly different from 1-h stimulation.

Because glucose phosphorylation is essential for glucose regulation of β-cell function, the importance of glucose phosphorylation and metabolism on the short-term regulation of SPHK activity was determined. An analog of glucose, 2-deoxy-D-glucose (2-DG), which is transported into β-cells and is phosphorylated by glucokinase but is metabolized too slowly to be an energy source for insulin secretion (30, 31), stimulated islet SPHK activity to levels similar to those observed with short-term glucose stimulation (**Fig. 2**). Another glucose analog, 3-o-methylglucose (3-oMeG), which is also transported into β-cells but is neither phosphorylated nor metabolized through glycolysis, failed to stimulate islet SPHK activity (Fig. 2).

Unlike glucose, α-ketoisocaproic acid (KIC), a metabolite of leucine that bypasses the glucose transporter, glucokinase, and glycolytic pathway and fuels the citric acid cycle through the formation of acetyl CoA, did not significantly activate islet SPHK after 1 h (Fig. 1A). In addition, glyceraldehyde (Gly), which stimulates metabolism by entering the glycolytic pathway directly, did not increase SPHK activity (Fig. 1B).

As changes in phosphate metabolism have been demonstrated to impact insulin-secretory effects in islets (32), orthophosphate was added to islets prior to glucose stimulation to address whether changes in intracellular inorganic phosphate impacted glucose-mediated SPHK activation. There was no significant effect of orthophosphate addition on basal islet SPHK activity (**Fig. 3**). However, the addition of orthophosphate prevented the stimulatory effect of glucose on SPHK activity (Fig. 3).

Effect of long-term glucose stimulation on SPHK expression and activity

To determine the effect of long-term glucose stimulation on β-cell SPHK activity, isolated islets were cultured for 7 days at 5.5 mM or 11 mM glucose. Islets cultured at 11 mM glucose showed a 6-fold increase in SPHK activity compared with islets cultured at the basal glucose concentration (**Fig. 4**). Basal islet SPHK activity following 7 days of culture was similar to that in isolated islets cultured overnight in 5.5 mM glucose (Fig. 4).

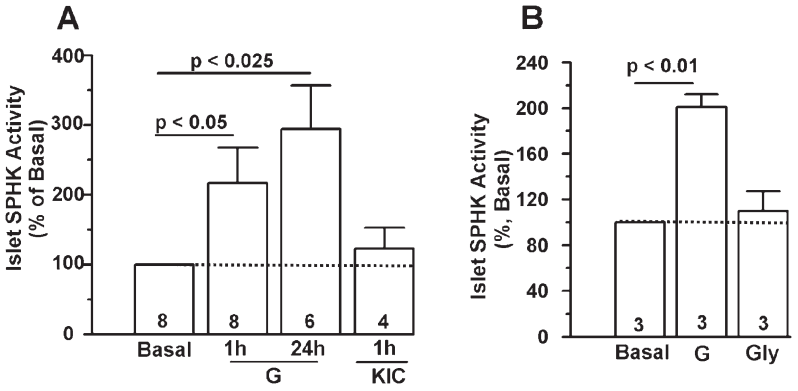

Fig. 1. SPHK activation by glucose is rapid and independent of ATP generation. Islets were cultured (A) 1 h in KRBH, or 24 h in CMRL-1066 containing glucose (G; 11 mM), or 1 h in α-ketoisocaproic acid (KIC; 11 mM), as indicated; or (B) 1 h in KRBH with glyceraldehyde (Gly; 11 mM). Sphingosine phosphorylation by SPHK in cell extracts was determined. Values are means of percent basal ± SE for the number of independent determinations shown at the base of each bar. Basal SPHK activity was 2.51 ± 0.68 pmole S1P/min/mg total protein. KRBH, Krebs Ringer bicarbonate HEPES; SPHK, sphingosine kinase; S1P, sphingosine 1-phosphate.

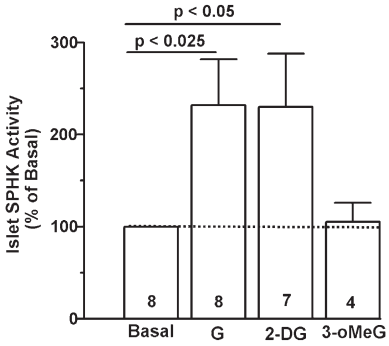

Fig. 2. SPHK activation by glucose is dependent on hexose phosphorylation. Islets were incubated for 1 h in KRBH containing glucose (G; 11 mM), 2-deoxyglucose (2-DG; 11 mM), or 3-o-methylglucose (3-oMeG; 11 mM). Sphingosine phosphorylation by SPHK in cell extracts was determined. Values are means of percent basal ± SE for independent determinations shown at the base of each bar. Basal SPHK activity was 3.71 ± 0.81 pmole S1P/min/mg total protein. SPHK, sphingosine kinase; S1P, sphingosine 1-phosphate.

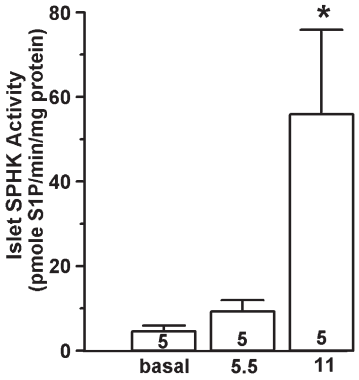

Fig. 4. Effect of long-term glucose stimulation on SPHK activity in islets. Islets were cultured for 7 days in CMRL-1066 containing either 5.5 mM or 11 mM glucose (G). Basal values represent islets cultured overnight in 5.5 mM G. Sphingosine phosphorylation by SPHK in cell extracts was determined. Values are means ± SE for number of independent experiments shown at the base of each bar. *$P \leq 0.05$ versus basal and 7 days 5.5 mM G. SPHK, sphingosine kinase.

Glucose regulation of SPHK gene and protein expression in pancreatic islets was also investigated. Although SPHK2 activity predominates in β-cells, mRNAs for both SPHK1a and SPHK2 are expressed in rat islets (11). SPHK1a mRNA in islets treated for 7 days at 11 mM glucose was increased by about 2.3-fold over that of freshly isolated islets and 2-fold over islets cultured at the basal glucose concentration (**Fig. 5A**). The increase in SPHK1a mRNA was not affected by treatment with Ara-C, indicating that cell proliferation was not a contributing factor to the increase in message at higher glucose concentrations. There was no significant change in SPHK1a mRNA in islets cultured at the basal glucose concentration compared with fresh islets (Fig. 5A). Similar to SPHK1a mRNA, SPHK2 mRNA levels in islets cultured for 7 days under hyperglycemic conditions were also significantly increased over the levels in freshly isolated islets and islets cultured under basal glucose conditions (Fig. 5B). Treatment with Ara-C resulted in a modest, but not statistically significant, decrease in SPHK2 mRNA in islets cultured at both 5.5 and 11 mM glucose. Although treatment with high glucose resulted in increased mRNA levels for both SPHK1 and SPHK2 in islets, there was no increase in SPHK1 or SPHK2 protein expression following long-term culture of islets in high glucose (Fig. 5C, D). In fact, SPHK1 protein expression in islets cultured in hyperglycemic conditions decreased by approximately 42% compared with fresh islets (Fig. 5C).

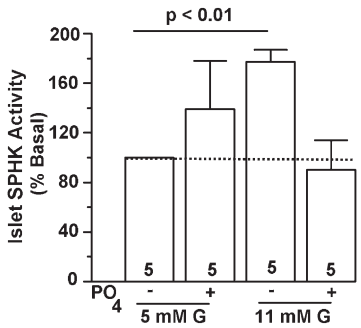

Fig. 3. Inorganic phosphate blocks glucose-stimulated SPHK activation. Islets were preincubated for 30 min in KRBH containing 5.5 mM glucose (G) in the absence (−) or presence (+) of 1.5 mM Na_2HPO_4 (PO_4), followed by 1 h incubation in KRBH containing either 5.5 mM G or 11 mM G in the absence or presence of PO_4. Sphingosine phosphorylation by SPHK in cell extracts was determined. Values are means of percent basal ± SE for the number of independent determinations indicated. Basal SPHK activity (5 mM G (−)PO_4) was 3.19 ± 0.47 pmole S1P/min/mg total protein. KRBH, Krebs Ringer bicarbonate HEPES; SPHK, sphingosine kinase; S1P, sphingosine 1-phosphate.

Long-term effects of glucose on SPHK activity, mRNA levels, and isozyme expression were evaluated in glucose-responsive INS-1e insulinoma cells. INS-1e cells are normally cultured at 11 mM glucose to promote growth. To study the effect of altering glucose concentrations in this model, INS-1e cells were first cultured at a lower glucose concentration (5.5 mM) to upregulate glucose-responsive pathways (33) and then cultured at higher glucose concentrations for 4 days. INS-1e cell SPHK activity increased in response to 17 mM glucose, while SPHK activity in cells cultured at 5.5 mM and 11 mM glucose was not increased over that of cells cultured in standard media (**Fig. 6**). To determine whether increased SPHK activity in these cells was the result of increased expression of SPHK isoforms, parallel cultures were analyzed by Western blot using SPHK-specific antibodies. SPHK1 and SPHK2 protein expression was not altered in response to changes in glucose concentration (**Fig.7A**, B). In contrast to the effects of hyperglycemia on SPHK1 and SPHK2 mRNAs in islets, there was no increase in message for either isoform in INS-1e cells (data not shown).

To address the findings that SPHK activity (islets and INS-1e cells) and SPHK message (islets) increase in response to long-term glucose treatment while SPHK protein levels are not altered, the stability of each isoform was assessed by Western blot analysis. INS-1e cells were cul-

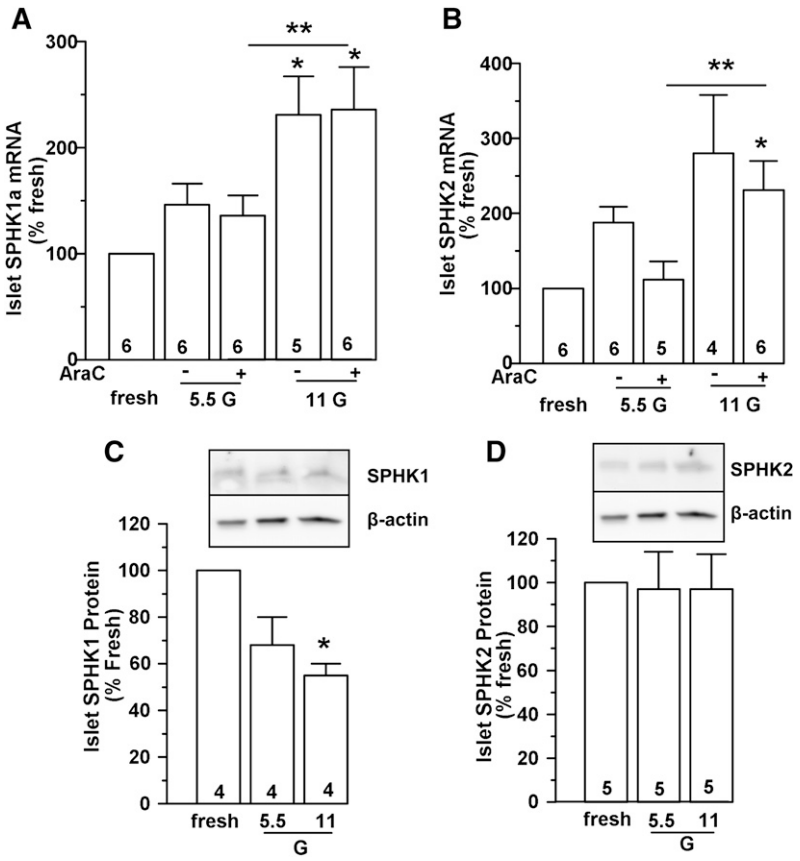

Fig. 5. Effect of long-term glucose stimulation on islet SPHK mRNA and protein levels . Islets were cultured for 7 days in media containing either 5.5 mM or 11 mM glucose (G) and the presence (+) or absence (−) of 10 μM AraC. Fresh islets were harvested immediately after pancreatic isolation. SPHK mRNA: Islet levels of mRNA relative to 18S rRNA for SPHK1a (A) and SPHK2 (B) were determined. SPHK mRNA levels are expressed as % change from fresh islets, as determined by QPCR. Values are means ± SE for 4–6 independent experiments. *$P \leqslant 0.025$ versus fresh islets, **$P \leqslant 0.05$ versus 5.5 mM G + AraC. SPHK Protein: Using Western blotting, SPHK1 (C) or SPHK2 (D) levels were normalized to β-actin. Values are mean % of fresh islet isoform expression ± SE for 4 (SPHK1) and 5 (SPHK2) independent experiments. * $P < 0.005$ versus fresh islets. Inset above each individual graph is a representative immunoblot for SPHK1 (C) and SPHK2 (D) protein levels relative to β-actin expression. SPHK, sphingosine kinase.

tured for 1–24 h in standard media containing 10 μM cycloheximide to block new protein synthesis. Western blot analysis demonstrated that SPHK1 expression was stable over the entire course of treatment, whereas SPHK2 expression decayed to 66% of control (**Fig. 8**). On the basis of these findings, the half-life of SPHK2 is estimated to be at least 41 h, while the half-life of SPHK1 could not be determined because there was no discernible decay in SPHK1 expression over the time course.

Small interfering RNA (siSPHK2) effect on SPHK2 expression and cell growth

SPHK2 activity was previously reported to be the predominant SPHK isoform activity in INS-1e insulinoma cells (11). The participation of SPHK2 in β-cell growth was investigated in INS-1e cells transfected with siSPHK2. Three days following transfection with either siLuc- or siSPHK2-containing plasmid, INS-1e cells were sorted by flow cytometry. SPHK2 mRNA levels from siSPHK2-transfected cells showed a reduction in SPHK2 mRNA levels to 9.6 ± 3.7% ($P < 0.05$) of levels in control siLUC-transfected cells. In GFP-negative sorted cells, SPHK2 mRNA levels were 121 ± 10% of control. In siSPHK2-transfected cells, SPHK2 protein levels were decreased to about 36% of control cells (**Fig. 9A**), and SPHK activity was reduced to 57 ± 11% ($P < 0.02$) of negative sort cells and to 58 ± 10% ($P < 0.01$) of untransfected control cells (Fig. 9B).

To determine the effects of SPHK2 on basal INS-1e cell growth, transfected and sorted siSPHK2 cells were cultured in media containing 11 mM glucose and assayed for DNA content. This concentration of glucose is necessary to support cell growth in this immortalized cell line. After 11 days, growth of siSPHK2-transfected cells was reduced by more than 51% compared with cells that were similarly transfected with siLuc or to untransfected (basal) cells (Fig. 9C). Cells were also cultured with exogenous S1P to determine if it could replace the loss of S1P coincident with reduced SPHK2 activity. The presence of exogenous S1P had no significant effect on cell growth in control or

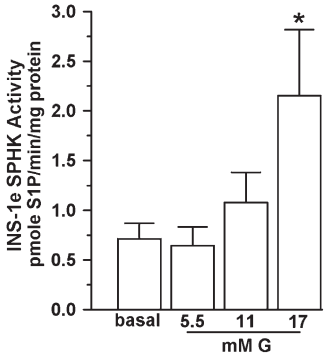

Fig. 6. SPHK activity is increased by high glucose in INS-1e cells. INS-1e cells were cultured for 2 weeks in RPMI-1640 containing 5.5 mM glucose. Cells were then cultured in either 5.5, 11, or 17 mM glucose (G) for 4 days. Cells were harvested in SPHK buffer, and cytosolic lysates were assayed for SPHK activity. Basal represents INS-1e cells cultured in standard media (11 mM G) throughout the entire period. Values are means ± SE for 10 experiments. *$P < 0.05$ versus basal and 5.5 mM G. SPHK, sphingosine kinase.

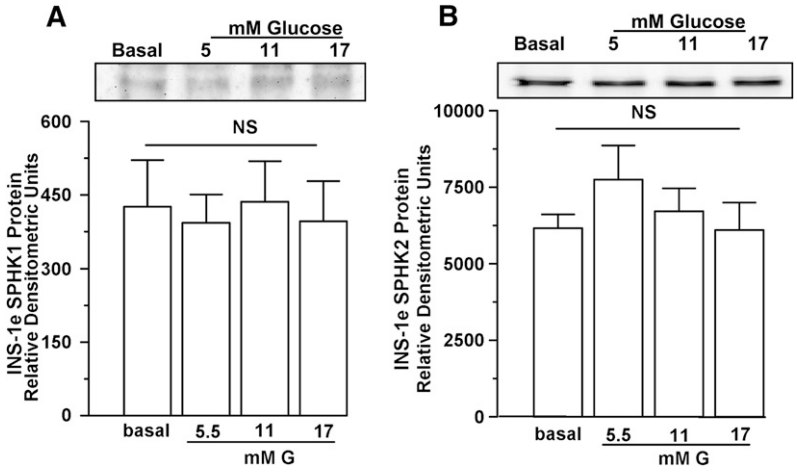

Fig. 7. SPHK isozyme levels are not altered by glucose. INS-1e cells were cultured for 2 weeks in RPMI-1640 containing 5.5 mM glucose. Cells were then cultured in 5.5, 11, or 17 mM glucose (G) for 4 days. Cells were harvested for Western blot analysis. Whole cell extracts (50–60 μg) were analyzed using SPHK1 (A) or SPHK2 (B) specific antibodies. Basal represents INS-1e cells grown in standard RPMI-1640 media (11 mM G) throughout the entire treatment period. Panel shows representative densitometric image for each antibody. Values are mean relative densitometric units ± SE for 8 (SPHK1) and 9 (SPHK2) independent experiments. SPHK, sphingosine kinase.

transfected cells (Fig. 9C). The effect of siSPHK2 transfection was also observed after only 5 days of culture when growth of INS-1e cells was reduced by 30 ± 11% ($P \leqslant 0.02$) of siLuc transfected cells.

DISCUSSION

We previously reported that SPHK activity is present in isolated rat islets and is activated by cytokines in a rapid and sustained manner, providing evidence that the enzyme is regulated and responsive to certain cytotoxic stimuli (11). Moreover, S1P inhibited the G_s-coupled adenylyl cyclase activation and insulin secretion induced by glucagon-like peptide-1 (7), and it showed protective effects against cytokine-induced apoptosis in isolated islets (8). Because glucose is a reported regulator of SPHK activity in vascular endothelial cells isolated from diabetic animals (34), we set out to determine if glucose is a regulator of islet SPHK using an in vitro model of chronic glucose exposure of isolated islets that mimics the effects of hyperglycemia in type 2 diabetes mellitus (16, 19). In the present study, when islets were cultured for 7 days at a normal physiological glucose level, basal SPHK activity was similar to the activity previously reported in freshly isolated islets (11). However, prolonged culture of islets under moderately hyperglycemic conditions (11 mM glucose) increased SPHK activity 2- to 3-fold above basal. Comparable results were also seen in INS-1e cells cultured in hyperglycemic conditions. Chronic glucose stimulation also increased SPHK1 and SPHK2 mRNA levels 1.5- to 2-fold over basal and freshly isolated islet levels. These results suggest that glucose contributes to the long-term regulation of SPHK activity. It is not known at this time whether glucose affects the stability of SPHK mRNAs or transcriptional activity of the genes. In addition, hyperglycemia increases β-cell proliferation (35), which might contribute to SPHK expression levels. To address this question, Ara-C was added to the islet cultures to inhibit cell growth. Treatment with Ara-C resulted in modest changes in SPHK2 mRNA levels in islets cultured for 7 days. Although this decrease may represent inhibition of β-cell (and other cell) proliferation, the data demonstrate a significant effect of hyperglycemia on SPHK1 and SPHK2 mRNA levels even in the presence of Ara-C, suggesting that cell proliferation is not sufficient to account for the effect of glucose on SPHK mRNA levels.

A novel finding of this study is the long half-life of the SPHK proteins. In light of this finding, the failure of increases in mRNA to be reflected in increased SPHK pro-

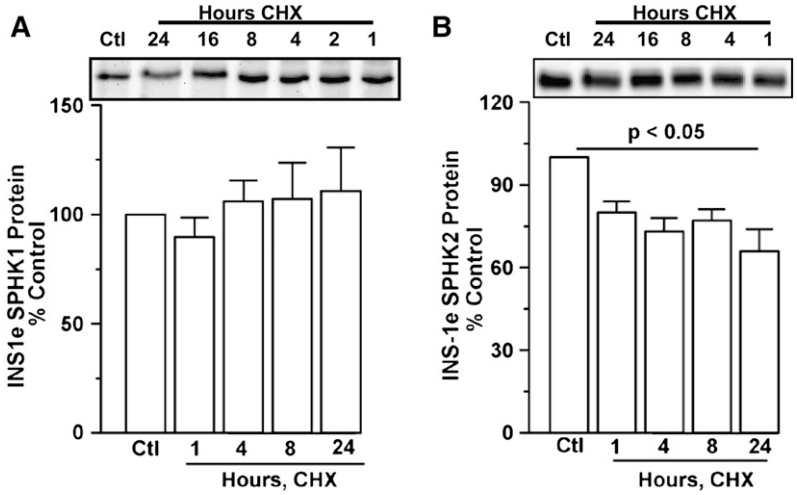

Fig. 8. SPHK1 and SPHK2 are stable proteins. INS-1e cells were cultured in standard RPMI media. At 80% confluence, cells were untreated (Ctl) or treated with cycloheximide (10 μM, CHX) for 1–24 h. Cells were harvested and whole cell extracts (50–60 μg) were analyzed using SPHK1 (A) or SPHK2 (B) specific antibodies. Inset shows representative densitometric image for Western blot of cells harvested at indicated time point following CHX treatment. Graph values are mean % control (Ctl) ± SE for 11 (SPHK1) and 3 (SPHK2) independent experiments. *$P < 0.05$ versus control at all time points. SPHK, sphingosine kinase.

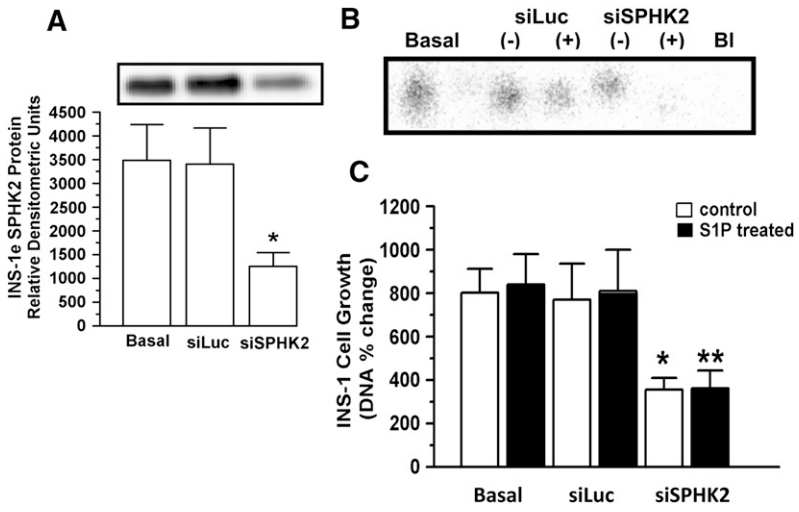

Fig. 9. Knock-down of SPHK2 blocks cellular growth. INS-1e cells were either not transfected (basal) or transfected with siLuciferase-pSUPER (siLuc) or siSPHK2-pSUPER (siSPHK2). Three days after transfection the cells were sorted by GFP-gated cytometry and used for Western blot analysis for SPHK2, SPHK activity assays, or cell growth in the presence or absence of S1P (400 nM). All studies were performed in RPMI containing 11 mM G. (A) SPHK2 protein levels for 6 independent experiments. $*P < 0.01$ versus basal and siLuc. Inset is representative immunoblot of SPHK2 protein levels for basal, siLuc, and siSPHK2 INS-1e extracts. (B) Representative phosphoimage of S1P extracted and isolated by thin-layer chromatography. SPHK activity was determined on cytosolic fractions (20 μg) from untransfected cells (basal), and cells collected from the negative (−) and positive (+) sort for GFP in siLuc and siSPHK2 transfected cells. Blank (Bl) represents S1P produced in the absence of added sphingosine substrate. (C) INS-1e cell growth as measured by DNA content. Cells were grown for 11 days in the absence (control) or presence of S1P (400 nM), as indicated. Values are % change in DNA compared with time-zero paired cells, and are means ± SE for 4 independent experiments. $*P \leq 0.02$, $**P \leq 0.05$ versus siLUC sorted cells. SPHK, sphingosine kinase; S1P, sphingosine 1-phosphate.

tein levels indicates that increases in SPHK activity in response to hyperglycemia are not dependent on increased enzyme expression alone, particularly with acute hyperglycemia. The current studies do not address whether long-term treatment of β-cells under hyperglycemic conditions alters the stability of SPHK isoforms or regulates translation or posttranslational mechanisms. We have demonstrated in INS-1e cells that phorbol esters acutely (1–4 h) activate SPHK (unpublished observations), suggesting that a posttranslational modification by protein kinase C plays a role in regulation of SPHK activity in β-cells. Future studies will address whether glucose modulates SPHK activity via covalent modifications in a similar fashion.

Glucose stimulation of β-cells has been reported to regulate the expression of multiple genes involved in metabolism, signaling, transcription, insulin secretion, and cell cycle regulation (36). Moreover, glucose stimulates DNA biosynthesis and cell proliferation in adult rat islets (37). Not only long-term but also short-term effects (min or h) of glucose on gene transcription have been reported for islets and INS-1e cells (7, 13). Glucose also had short-term effects on SPHK activity within 1 h and 24 h that are not attributable to new protein synthesis. The regulation of SPHK activity has been linked to growth factors (38, 39), protein kinase C (40), and protein kinase A (41), among other cellular mediators. Agonist-induced activation of SPHK1 has been demonstrated to be mediated through ERK1/2 kinase activity at specific serine residues (42), and PKC-activating phorbol esters induce SPHK (43). As glucose stimulates islet ERK 1/2 activation (13) and can activate PKC and cyclic AMP generation/protein kinase A (22), SPHK activation in islets may be at least partially mediated through one of these signaling pathways. It appears that maximal glucose-stimulated SPHK activation is achieved within 24 h as the fold-activation was similar to that observed in islets cultured for 1 week at the high glucose concentration. Although glucose has the potential to modulate intracellular S1P levels through inhibition of S1P phosphohydrolase (44) or S1P lyase (45) activities in addition to increasing SPHK activity, the ex vivo assay described herein is designed to inhibit degradation of S1P through the use of phosphatase inhibitors. Therefore, this question cannot be addressed by our studies.

The mechanism by which glucose induces the rapid change in islet SPHK activity was investigated with several nutrient and nonnutritive agents. The leucine metabolite KIC is metabolized and generates acetyl CoA that enters the citric acid cycle and contributes to rapid generation of ATP with subsequent β-cell depolarization and insulin secretion (46). However, KIC at a concentration known to fuel a rapid and robust insulin secretory response (47) failed to stimulate SPHK activity. Thus, early changes in SPHK activation by glucose appear not to depend upon ATP generation or depolarization-dependent Ca^{2+} influx associated with KIC actions on β-cells (48).

Glucokinase is the glucose sensor in β-cell glucose-mediated insulin secretion (49), and this step in glucose metabolism was investigated for effects on SPHK activity. 2-DG is a glucose analog that is readily phosphorylated by glucokinase, but it does not stimulate insulin secretion because of failure to be further metabolized through glycolysis (31). In the short term, 2-DG stimulated islet SPHK activity to levels similar to glucose stimulation. In contrast, 3-oMeG, an analog that is transported but neither phosphorylated nor metabolized, failed to stimulate SPHK activity. These results suggested that glucose transport and phosphorylation are necessary and sufficient for acute SPHK activation.

While glyceraldehyde is known to be an insulin secretagogue, the mechanism for this response has been debated for some time. Recent elegant metabolic studies demonstrated that glyceraldehyde is efficiently metabolized to carbon dioxide through triokinase activity, followed by activation of glyceraldehyde phosphate dehydrogenase activity and increased cytosolic NADH levels (50). Equimolar triose equivalents of glyceraldehyde led to increased insulin secretion compared with glucose, yet ATP production was much lower than that of glucose (51). The failure of glyceraldehyde to activate SPHK indicates that glycolysis, altered NAD+/NADH, or downstream effects of glucose metabolites on ATP generation are not responsible for the acute stimulation of SPHK by glucose.

The requirement of glucose phosphorylation for SPHK activation raises the question of whether intracellular phosphate depletion might play a role in enzyme regulation. It has long been appreciated that metabolic secretagogue-induced insulin release is accompanied by declines in intracellular inorganic phosphate, either as a result of a phenomenon known as the phosphate flush, the rapid efflux of orthophosphate from islets following stimulation of insulin secretion by glucose (52), or through trapping of phosphate in metabolites and phosphorylated end products. Glucose analogs, such as 2-DG, decrease intracellular inorganic phosphate and have been demonstrated to inhibit cellular respiration in ascites tumor cells and to potentiate KIC-induced insulin secretion in β-cells (32, 53). These effects are abrogated when cytoplasmic orthophosphate levels are maintained (32). In islets, the presence of orthophosphate inhibited the stimulatory effect of glucose on SPHK activity. Orthophosphate has been described as having stimulatory effects on islet glutaminase, which catalyzes the conversion of glutamine to glutamate (54). Glutamine is an amino acid that does not stimulate insulin release directly; rather, it provides anaplerotic input through metabolism to glutamate and α-ketoglutarate (55). Thus, there is precedent for intracellular orthophosphate levels to regulate key glucose/metabolic pathways, although there is no known link between SPHK regulation and glutamine levels in cells. These data suggest that changes in intracellular inorganic phosphate concentrations represent a novel regulator of SPHK activity.

A role for SPHK2 expression and activity in β-cell growth under growth-sustaining glucose concentrations is an important and novel observation. SPHK2 has been reported to localize in part to the nuclei of cells (9) where it mediates the activity of platelet-derived growth factor (56) or inhibits DNA biosynthesis when over-expressed (9). The lack of effect of extracellular S1P on insulinoma cell mitogenesis suggests that plasma membrane S1PRs not only do not mediate autacoid responses to the intracellular changes in S1P production in β-cells but also that S1P provided in vivo in serum (57) at concentrations similar to that provided in vitro in this study do not appear to modulate β-cell growth. This laboratory recently reported that exogenous S1P in the absence of serum protects β-cells from apoptosis (8). However, the lack of effect of exogenous S1P on cell growth in the present study suggests that apoptosis plays little if any role given the caveat that serum proteins with the potential to bind S1P were present. Rather, intracellular generation of S1P appears to affect β-cell mitogenesis as the knockdown of SPHK2 mRNA, protein, and activity corresponded with changes in INS-1e cell growth. Alternatively, the reduced conversion of sphingosine to S1P in siSPHK2-treated cells might result in an elevated sphingosine content that could inhibit cell growth (58). S1P stimulates mitogenesis in fibroblasts via increased DNA binding activity of activator protein-1 (AP-1) (59) and promotes Swiss 3T3 cell growth through activation of the mitogen-activated protein kinase pathway (60) and cyclin-dependent serine/threonine kinases cdk2 and p34cdc2 (61). Therefore, it is probable that β-cell mitogenesis is modulated by similar mechanisms in response to endogenous S1P production. Future studies will explore these possibilities.

CONCLUSION

Glucose exerted regulatory effects on SPHK expression and activity in islets and a homogeneous population of insulinoma cells. SPHK appears sensitive to changes in cellular phosphate levels rather than glycolytic or oxidative changes. Glucose stimulation of islets results in prolonged upregulation of SPHK activity that appears to modulate β-cell mitogenesis.

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