Sphingosine Kinase Activity and Sphingosine-1 Phosphate Production in Rat Pancreatic Islets and INS-1 Cells

Response to Cytokines

Lucy D. Mastrandrea,[1,2] Shawn M. Sessanna,[1] and Suzanne G. Laychock[1]

Sphingosine-1 phosphate (S1P) is a bioactive sphingolipid with the potential to mobilize Ca^{2+}, to inhibit apoptosis, and to promote mitogenesis. Sphingosine kinase (SPHK) and S1P were characterized in INS-1 insulinoma cells and isolated rat islets of Langerhans. SPHK activity increased in INS-1 cell homogenates treated with interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α), and responses were additive. IL-1β or TNF-α increased islet SPHK activity within 15 min to 1 h; activity remained elevated after 8 h. SPHK2 was the predominant active isoform in INS-1 cells; little or no SPHK1 activity was detected. Cytokines increased endogenous S1P biosynthesis in $^{32}P_i$-prelabeled INS-1 cells, and cycloheximide inhibited the response after 8 h, suggesting that protein synthesis mediated the response. There was no [^{32}P]S1P release from cells. Compared with basal values, IL-1β and TNF-α induced increases in SPHK1a mRNA levels relative to 18S ribosomal RNA in INS-1 cells within 1 h; relative SPHK2 mRNA levels were unchanged after cytokine treatment. IL-1β, but not TNF-α, induced relative SPHK1a mRNA expression levels within 1 h in islets, whereas SPHK2 mRNA levels were unchanged. Thus, IL-1β and TNF-α induced an early and sustained increase in SPHK activity in INS-1 cells and isolated islets, suggesting that S1P plays a role in the pathological response of pancreatic β-cells to cytokines. *Diabetes* 54:1429–1436, 2005

Sphingolipids are necessary for maintaining cellular membrane structure and integrity. In addition to their structural role, metabolites of sphingomyelin act as a novel class of lipid second messenger mediating a variety of cellular activities, including growth, differentiation, motility, and apoptosis (1–4). Sphingosine-1 phosphate (S1P) acts as an intracellular second messenger and as a cell surface receptor ligand (5). S1P stimulates DNA synthesis, calcium mobilization, and mitogen-activated protein kinase activation (6,7) and promotes cell proliferation in opposition to apoptosis in several cell types (8,9). Extracellular S1P binds to a family of receptor isoforms in the endothelial differentiation gene–encoded (EDG or S1P) receptor class (7,8). Lysophosphatidic acid preferentially binds to other select EDG receptors (10). This laboratory recently identified EDG receptor mRNAs in rat and mouse pancreatic islets and INS-1 cells (11).

Sphingosine kinase (SPHK) catalyzes S1P biosynthesis from sphingosine. Two isozymes of SPHK (types 1 and 2) have been cloned (12–14). The two enzymes are similar in amino acid sequence (12), sharing five conserved domains involved in catalysis of sphingosine phosphorylation (15). SPHK1 is a primarily cytosolic enzyme stimulated by growth factors, such as platelet-derived growth factor and serum (16,17), nerve growth factor (18), protein kinase C (PKC) (19,20), vitamin D_3 (21), protein kinase A (22), binding of carbachol to m2 and m3 muscarinic receptors (16), and tumor necrosis factor-α (TNF-α) (17,23). Moreover, SPHK1 overexpression in PC12 cells increased S1P levels and suppressed ceramide-induced apoptosis by reducing caspase and stress-activated protein kinase/c-Jun NH_2-terminal kinase activities (24). Whereas SPHK1 has been described primarily as involved in growth-promoting and antiapoptotic activities, SPHK2 is reported to be involved in apoptosis induction and inhibition of DNA synthesis/cell growth (25,26). Thus, the SPHK isoforms, both of which catalyze S1P formation, appear to have different cellular signaling functions.

Cytokines are implicated in inducing or promoting the development of type 1 diabetes. Interferon-γ, interleukin-1β (IL-1β), and TNF-α (27) inhibit β-cell metabolism and insulin secretion (28) and induce nitric oxide (NO) production (29) and apoptosis (30). The present study determined the capacity of β-cells to synthesize S1P and evaluated the regulation of the S1P pathway in pancreatic islet cells subjected to cytokine-induced stress. The results demonstrate for the first time that the SPHK pathway is active in pancreatic β-cells and that it is induced in response to cytotoxic stress.

From the [1]Department of Pharmacology and Toxicology, The State University of New York, Buffalo, New York; and the [2]Department of Pediatrics, School of Medicine and Biomedical Sciences, The State University of New York, Buffalo, New York.

Address correspondence and reprint requests to Suzanne G. Laychock, 102 Farber Hall, The State University of New York at Buffalo, School of Medicine, 3435 Main St., Buffalo, NY 14214. E-mail: laychock@buffalo.edu.

Received for publication 19 August 2004 and accepted in revised form 28 January 2005.

EDG, endothelial differentiation gene–encoded receptor; FAF-BSA, fatty acid–free BSA; FBS, fetal bovine serum; IL-1β, interleukin-1β; PKC, protein kinase C; S1P, sphingosine-1 phosphate; SPHK sphingosine kinase; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor-α.

RESEARCH DESIGN AND METHODS

S1P and sphingosine were from Biomol Research Laboratory (Plymouth Meeting, PA). [γ-^{32}P]ATP (3,000 Ci/mmol) and [^{32}P]orthophosphate (8,500–9,120 Ci/mmol) were from Perkin Elmer (Boston, MA). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Human recombinant IL-1β, TNF-α, and interferon-γ were obtained from R&D Systems (Minneapolis, MN). Culture media and molecular biology reagents were from Invitrogen (Grand Island, NY). QuantumRNA Classic 18S standards (488 bp) were from Ambion (Austin, TX). BSA, essentially fatty acid free, fraction V (FAF-BSA), ATP, and other reagents were from Sigma Chemical (St. Louis, MO).

Cell and tissue preparation. Rat pancreatic islets were isolated as described previously (31). All animal procedures were approved by the Institutional Animal Care and Use Committee. Islets were cultured in CMRL-1066 medium containing 9% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml), for up to 24 h in the absence or presence of various stimuli indicated in the text. Cells of the rat insulinoma cell line, INS-1e (a gift of Dr. Claes Wollheim, Geneva, Switzerland) were cultured in modified RPMI-1640 (32); for 24 h before and during the experimental treatments, the INS-1 cells were cultured in medium in the absence of serum and the presence of 0.1% FAF-BSA.

mRNA quantitation. Levels of SPHK mRNA were determined by semiquantitative RT-PCR (11). Islet (15–20 islets per extract) RNA was extracted essentially as described previously (11). PCR was carried out using primers to SPHK1a: sense, 5′-AGCCACCTTCAAGGAGTGAC-3′; and antisense, 5′-CAGTCTGCTGGTTGCATAGC-3′ (319 bp). PCR was carried out using primers to SPHK2: sense, 5′-CCAGGCTGCTCCTATTGGTC-3′; and antisense, 5′-TTGAGCAACAGGTCAACACC-3′ (367 bp). PCR was carried out and quantitated as described previously (11) with 0.5 μl of Quantum RNA Classic 18S rRNA primers and 2 units of Platinum *pfx* DNA polymerase (SPHK1a) or *Taq* polymerase (SPHK2), 1.5 mmol/l $MgSO_4$ (SPHK1a), 1.2 mmol/l $MgCl_2$ (SPHK2), and 6 μl of enhancer solution (SPHK1a). Typical reaction conditions were as follows: 94°C for 2 min for initial denaturation, followed by 28–38 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 68°C for 60 s, with a final extension at 68°C for 7 min. Cycle time to achieve product linearity was determined in preliminary experiments. The image densities of SPHK mRNA and 18S rRNA were compared to determine the ratio of expression in experimental samples. PCR product identity was confirmed by automated DNA sequencing (11) using appropriate primers described above.

Assay of SPHK activity. Cells (2×10^6 cells per 10- × 30-mm dish) were cultured in serum-free media for 24 h to deprive cells of S1P present in serum. Then cells were treated with cytokines for the times indicated. Islets and harvested cells were lysed by freeze-thawing in SPHK buffer (33), followed by brief sonication for islets. Lysates were centrifuged at 15,000*g* for 20 min at 4°C, and the protein concentration of the supernatant fraction was determined. SPHK activity assays included (unless otherwise indicated): 40–80 μg of protein per reaction, 50 μmol/l sphingosine complexed with FAF-BSA (4 mg/ml suspension), and [^{32}P]ATP (2.5 μCi, 1 mmol/l) containing $MgCl_2$ (10 mmol/l) (14). Reaction linearity was determined in preliminary assays. SPHK isozyme activities were determined in the presence of 400 mmol/l KCl or 1% Triton X-100, as described previously (12). Labeled S1P was extracted (33) and separated by thin-layer chromatography (TLC) on heat-activated silica gel G plates using a solvent system containing 1-butanol:methanol:acetic acid:water (80:20:10:20 [vol/vol]). [^{32}P]S1P was quantitated by phosphoimager densitometric analysis using Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA). Sample densities were converted to counts per minute (cpm). The authenticity of the S1P in the samples was tested by treating lipid extracts with potassium hydroxide to remove lipids other than S1P (1) and by ninhydrin staining of TLC-resolved S1P standard. SPHK specific activity was expressed as picomoles of S1P formed per minute per milligram of total protein.

Subcellular fractionation of INS-1 cells. Nuclei were prepared as described previously (34). Cells were washed, centrifuged briefly, and resuspended in buffer A (10 mmol/l HEPES, pH 7.5, 5 mmol/l $MgCl_2$, 15 mmol/l KCl, and 1 mmol/l phenylmethylsulfonyl fluoride) at 5×10^6 cells/ml. The cells were then frozen in liquid nitrogen, thawed rapidly at 37°C, and passed through a 25-gauge needle 15 times. The homogenate was layered on top of a 500-μl sucrose cushion (50% sucrose in buffer A) and centrifuged for 5 min at 15,000*g*. Nuclei-free cytoplasm was removed from above the sucrose layer. Intact nuclei were resuspended in 200 μl of SPHK buffer (see above). The nuclei-free cytoplasm was then centrifuged at 100,000*g* for 60 min, and the pelleted membrane fraction was resuspended in 200 μl of SPHK buffer; the supernatant composed the cytosolic fraction.

Generation of stable murine SPHK1a transfectants and measurement of SPHK1 activity. INS-1 cells in serum- and antibiotic-free RPMI-1640 were transfected with 200 ng of pcDNA3.1-mSPHK1a vector (generous gift of Dr. Sarah Spiegel, Medical College of Virginia, Richmond, VA) or empty vector (generated by BSTX1 digestion of pcDNA3.1-mSPHK1a vector with subsequent religation), 1 μl of LipofectAMINE reagent, and 4 μl of LipofectAMINE PLUS reagent per well. Stable clones were established and assayed for SPHK activity as described above.

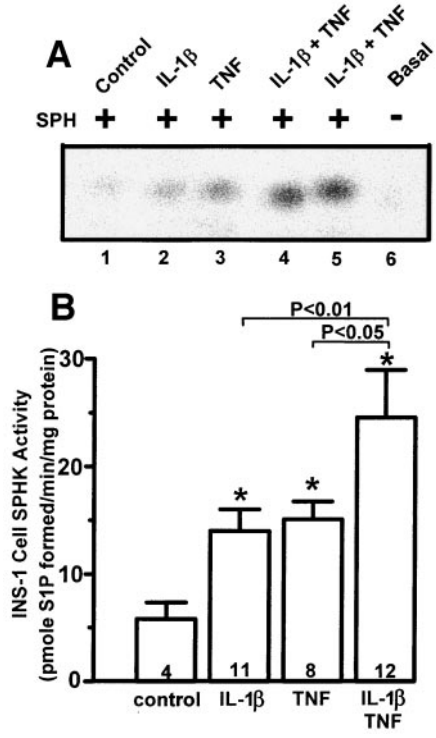

FIG. 1. SPHK activity in cytokine-treated INS-1 cells. INS-1 cells were cultured in the absence (control) or presence of IL-1β (2 ng/ml) and/or TNF-α (20 ng/ml) for 8 h. *A*: Representative phosphoimage of S1P produced, extracted, and isolated by TLC. SPHK activity was determined in the absence (−) and presence (+) of sphingosine (SPH, 50 μmol/l). *B*: INS-1 cell SPHK activity. Values are means ± SE for the number of independent experiments shown at the base of each bar. *P* values were determined by one-way ANOVA and multiple comparison test. **P* < 0.01 vs. control with sphingosine added.

Measurement of S1P production in intact cells. INS-1 cells (2×10^6 cells per 10- × 30-mm dish) were prelabeled with [^{32}P]orthophosphate (40 μCi/ml) for 24 h and then treated with agents indicated in the text. After washing with PBS, cells were lysed with acidic methanol (1 ml of methanol + 10 μl of 3 N HCl). [^{32}P]S1P was extracted in chloroform:1 mol/l sodium chloride:3 N sodium hydroxide (2:1:0.1 [vol/vol/vol]) (9). [^{32}P]S1P was resolved by TLC as described above.

Statistical analysis. Values are means ± SE. Significant differences between treatment groups were determined by Student's *t* test (paired) or one-way ANOVA with post hoc analysis using the Student-Newman-Keuls multiple-comparison test. Values of $P \leq 0.05$ were accepted as significant.

RESULTS

Regulation of SPHK activity in β-cells. Total SPHK activity, quantitated as the phosphorylation of sphingosine to S1P, was determined in INS-1 cell homogenate cytosolic fractions after treatment of intact cells with cytokines for 8 h. Cytosolic fractions were initially chosen for enzyme activity characterization because membrane-associated enzyme has been reported to be unstable (34). Control SPHK activity in samples containing sphingosine substrate was detectable above basal activity in samples lacking sphingosine (Fig. 1*A*, *lanes 1* and *6*). IL-1β, at a concentration (2 ng/ml) within the range previously reported to induce apoptosis in rat β-cells (35,36), significantly increased the SPHK activity in INS-1 cells by about 2.5-fold versus control with sphingosine (Fig. 1*A*, *lanes 1* and *2*,

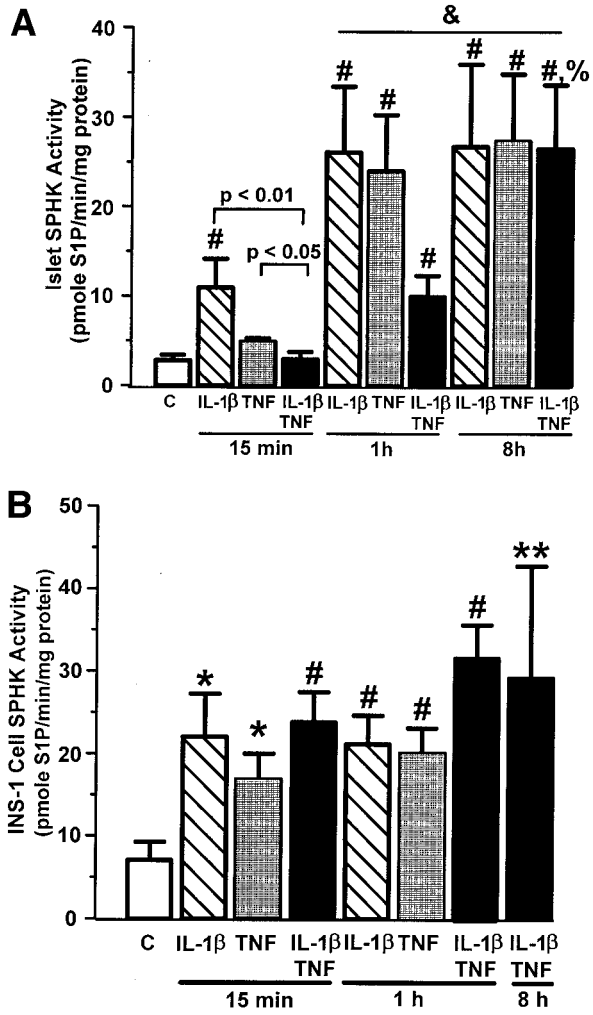

FIG. 2. SPHK activity in isolated rat islets and INS-1 cells. Islets (*A*) (5.5 mmol/l glucose) or INS-1 cells (*B*) (11 mmol/l glucose) were cultured in the absence (control, C) or presence of IL-1β (2 ng/ml) and/or TNF-α (20 ng/ml) for the times indicated. SPHK activity was determined in cell cytosolic fractions. Values are means ± SE for four to six (*A*) (controls, $n = 17$) and three to eight (*B*) determinations. *P* values were determined by one-way ANOVA and multiple comparison test. *$P < 0.01$, **$P < 0.02$, #$P < 0.001$ vs. control; &$P < 0.05$ vs. similarly treated islet groups at 15 min; %$P \leq 0.05$ vs. IL-1β + TNF-α, 1 h.

and *B*). At 2 ng/ml IL-1β, the stimulation of SPHK activity (335 ± 53% of control) was maximal, because SPHK activity at 0.1 ng/ml IL-1β (222 ± 59% of control) was not significantly different ($P > 0.05$). Treatment of cells with an apoptosis-inducing maximal concentration of TNF-α (37) resulted in a significant increase in SPHK activity to a level equivalent to that observed in IL-1β–treated cells (Fig. 1*A*, *lanes 1* and *3*, and *B*). There was no difference in INS-1 cell SPHK activity between 1 and 20 ng/ml TNF-α (data not shown). When IL-1β and TNF-α treatments were combined, SPHK activity increased significantly in the INS-1 cells versus control cells (Fig. 1*A*, *lanes 1*, *4*, and *5*, and *B*). This treatment also significantly increased activity versus either cytokine alone (Fig. 1*A*, *lanes 2–5*, and *B*).

Islets also showed significant and sustained increases in SPHK activity after culture with IL-1β for 15 min, 1 h, or 8 h (Fig. 2*A*). Similar responses were observed in islets cultured with TNF-α for 1 or 8 h (Fig. 2*A*). SPHK activation appeared maximal after 1 h of stimulation with IL-1β or TNF-α because activity was not increased further after 8 h (Fig. 2*A*). SPHK activity in islets treated with IL-1β plus TNF-α for 15 min was significantly lower than activity in islets treated with either cytokine alone; however, the activity increased in a time-dependent manner for as long as 8 h (Fig. 2*A*) and was significantly higher than control activity levels after 1 and 8 h (Fig. 2*A*).

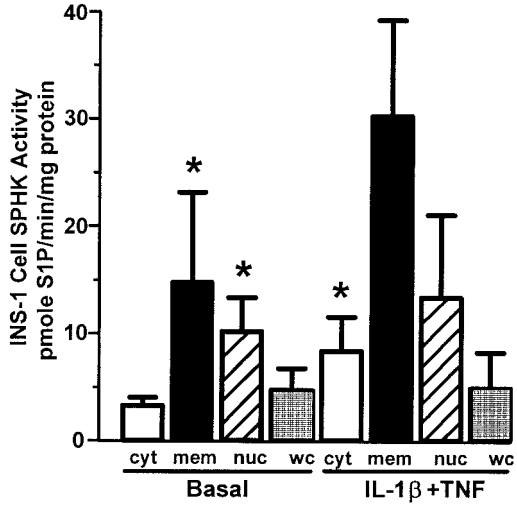

FIG. 3. SPHK activity in cytosolic (cyt), membrane (mem), and nuclear (nuc) subcellular fractions and in whole-cell (wc) homogenate. INS-1 cells were cultured in the absence (control) or presence of IL-1β (2 ng/ml) and TNF-α (20 ng/ml) overnight. Cytosolic, membrane, nuclear, and whole-cell preparations were assayed for SPHK activity. Values are means ± SE ($n = 3–5$). *P* values were determined by one-way ANOVA and multiple comparison test. *$P < 0.05$ vs. basal cytosolic enzyme activity.

INS-1 cells also showed rapid SPHK activation after culture with either IL-1β, TNF-α, or the combination of these cytokines (Fig. 2*B*). SPHK activation by the cytokines appeared to be maximal within 15 min because activity was not significantly higher after 1 h with either or both cytokines or after 8 h with the combined stimulus (Fig. 2*B*). No additivity in the responses to IL-1β or TNF-α was apparent at 15-min and 1-h treatment times (Fig. 2*B*).

The effects of cytokine stimulation on SPHK activity in subcellular fractions of INS-1 cells was also determined. In control cells, basal SPHK activity was approximately two- to threefold enriched in the particulate membrane and nuclear fractions of cells compared with the high-speed cytosolic fraction and the activity in whole-cell homogenates (Fig. 3). SPHK activity in the cytosolic fractions was not significantly different from those observed in the paired whole-cell homogenate preparations. After treatment of INS-1 cells with IL-1β and TNF-α for 18 h, there was a significant increase in SPHK activity in the cytosolic fraction compared with basal control cytosolic activity (Fig. 3). However, whereas SPHK activity in the particulate membrane and nuclear fractions of cytokine-stimulated cells exhibited a trend toward higher activity, the activity in these fractions was more variable than the cytosolic or whole-cell preparations and was not significantly different from the basal activity in comparable fractions isolated from control cells (Fig. 3). Moreover, no significant differences in SPHK activity were observed in whole-cell homogenates of cytokine-treated and untreated cells (Fig. 3).

SPHK isozymes in β-cells. The contribution of isozymes

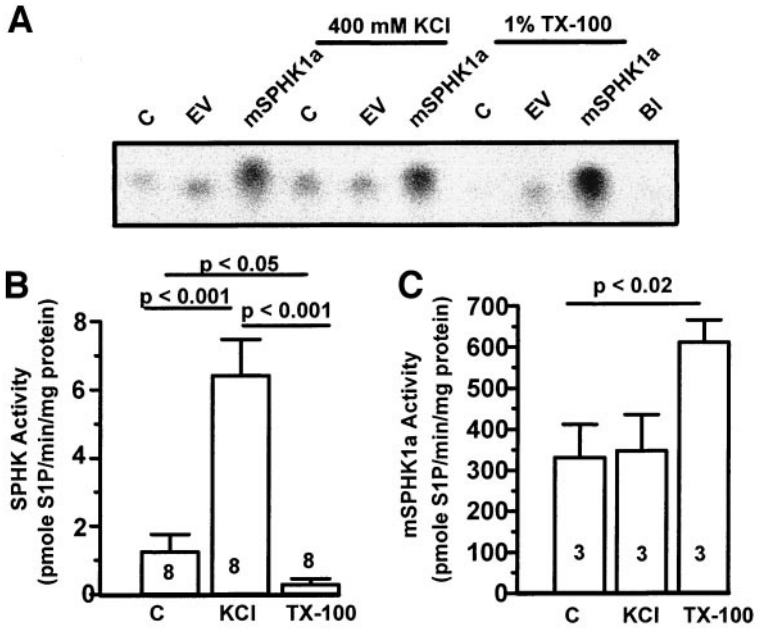

FIG. 4. SPHK2 is the predominant isozyme in INS-1 cells. SPHK activity in INS-1 cells, INS-1 cells stably transfected with empty pcDNA3.1 vector (EV), and INS-1 cell clonal population overexpressing recombinant mouse SPHK1a (mSPHK1a) was determined in the absence or presence of 400 mmol/l KCl and 1% Triton X-100. *A*: Representative phosphoimage of S1P extracted and isolated by TLC. *B*: Nontransfected INS-1 cell SPHK activity. *C*: mSPHK1a activity. Values are means ± SE for the number of independent experiments shown at the base of each bar. **P* values were determined by one-way ANOVA and multiple comparison test. C, control.

SPHK1 and SPHK2 to total SPHK activity in β-cells was determined by comparison of their activities in the presence of high salt concentration (KCl) and detergent (Triton X-100) (12). SPHK1 activity is inhibited by high salt concentration and activated by Triton X-100, whereas SPHK2 behaves in a reciprocal manner. INS-1 cell SPHK activity was activated in KCl-containing buffer by approximately sixfold, whereas the activity was almost undetectable in the presence of Triton X-100 (Fig. 4*A* and *B*), which is consistent with SPHK2 activity. In comparison, the effects of KCl and Triton X-100 on SPHK1 activity were assessed in INS-1 cells transfected with recombinant murine SPHK1a. Transfected INS-1 cells showed a significant activation of activity after treatment with Triton X-100 (Fig. 4*A* and *C*). Unexpectedly, the recombinant SPHK1a activity was not inhibited by KCl (Fig. 4*C*). It is unlikely that the lack of an inhibitory effect of KCl in transfected cells was due to activation of endogenous SPHK2 activity because the relative SPHK2 activity in nontransfected cells was only increased by ~5 pmol S1P · min^{-1} · mg $protein^{-1}$ with KCl treatment (Fig. 4*B*), and in transfected cells, SPHK total activity was 331 ± 81 pmol · min^{-1} · mg $protein^{-1}$ (Fig. 4*C*). Molar concentrations of KCl may be required for maximal enzyme inhibition (12).

SPHK1 has been described as a predominantly cytosolic enzyme that can translocate to the nucleus and plasma membrane (34,38), whereas SPHK2 has a functional nuclear localization signal (26). In INS-1 subcellular fractionation studies, KCl-stimulated SPHK2 activity was identified in the membrane-free cytosolic, membrane, and nuclear fractions (Fig. 5). Consistent with data in Fig. 4, SPHK2 cytosolic activity was significantly activated by KCl treatment. SPHK activity in each subcellular fraction was inhibited by treatment with Triton X-100 (Fig. 5).

Although the predominant basal SPHK isozyme in INS-1 cells appeared to be SPHK2, there remained the possibility that the activation of SPHK activity by cytokines shown in Figs. 1 and 2 could be explained by an augmentation of SPHK1a and/or SPHK2 activities. To address this question, INS-1 cells were treated with IL-1β and TNF-α for 1, 8, and 24 h, and cytosolic SPHK activity was assayed in the presence of either KCl or Triton X-100. SPHK activity was significantly increased by cytokine treatment at each time point, and SPHK2 activity appeared to predominate, as indicated by complete inhibition by Triton X-100 (Fig. 6). Although basal SPHK activity was stimulated by KCl treatment, indicative of SPHK2, the stimulated level of SPHK activity induced by cytokine treatment at the various time points was not further activated by KCl (Fig. 6).

S1P biosynthesis in intact cells. Endogenous S1P production in INS-1 cells was determined after overnight equilibration with $^{32}P_i$. When prelabeled INS-1 cells were cultured with sphingosine for 20 min to act as an exogenous substrate for SPHK, S1P levels were significantly increased relative to untreated cells (Fig. 7*A* and *B*). The presence of sphingosine did not affect secreted [^{32}P]S1P levels in media (Fig. 7*A*), which were largely undetected. Treatment of $^{32}P_i$-prelabeled intact INS-1 cells with IL-1β plus TNF-α for 8 h induced a significant increase in intracellular [^{32}P]S1P levels of 152 ± 13% over control

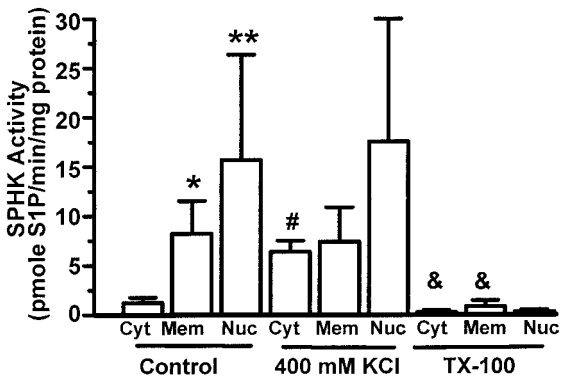

FIG. 5. Subcellular fractionation of INS-1 cell SPHK2 activity. INS-1 cell nuclear (Nuc), membrane (Mem), and cytosol (Cyt) fractions were prepared and assayed for SPHK activity in the absence (control) or presence of 400 mmol/l KCl or 1% Triton X-100. Values are means ± SE for *n* = 5–8. *P* values were determined by one-way ANOVA and multiple comparison test. **P* < 0.01, ***P* < 0.05, #*P* < 0.001 vs. cytosol control; &*P* < 0.05 vs. paired control fraction.

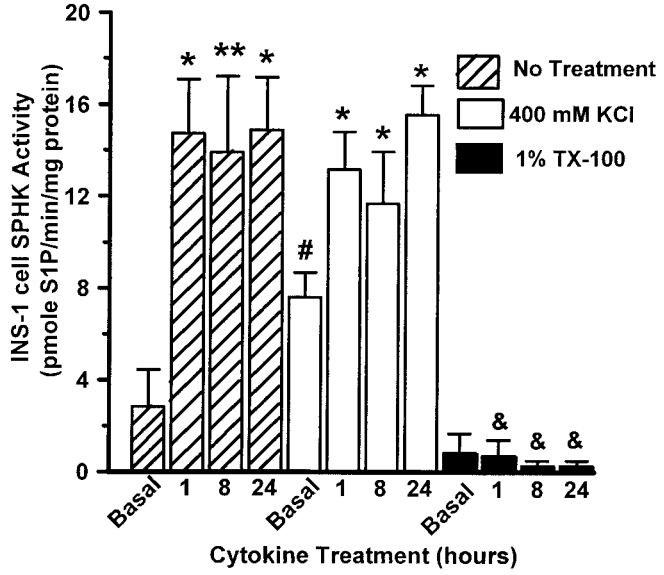

FIG. 6. SPHK2 is the predominant cytokine-activated isozyme in INS-1 cells. INS-1 cells were cultured in the absence (basal) or presence of IL-1β (2 ng/ml) and TNF-α (20 ng/ml) for the times indicated. SPHK activity was assayed in the absence or presence of 400 mmol/l KCl or 1% Triton X-100. Values are means ± SE (n = 3–4). P values were determined by one-way ANOVA and multiple comparison test. *P < 0.005, **P < 0.01 vs. similarly treated group basal value; #P < 0.05, &P < 0.005 vs. paired time untreated sample.

($P < 0.02$; Fig. 8). In contrast, ^{32}P-labeled total phospholipid levels in IL-1β– plus TNF-α–treated cells were only 120 ± 8% of control ($P < 0.05$) after 8 h (data not shown).

To determine whether new protein synthesis in part mediated the cytokine-induced change in S1P levels, INS-1 cells were cultured in the presence of cycloheximide

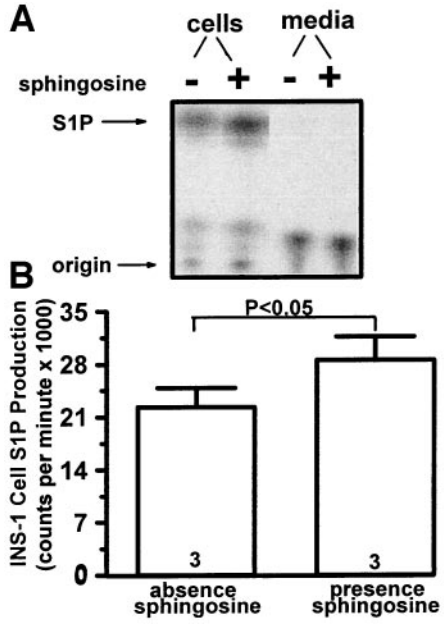

FIG. 7. Exogenous sphingosine is a substrate for SPHK. INS-1 cells were cultured with $^{32}P_i$ (40 μCi/ml) for 24 h. Before harvest (20 min), some cells were treated with sphingosine (50 μmol/l). S1P was extracted from intact cells or media and isolated by TLC. *A*: Representative phosphoimage from an experiment showing ^{32}P-S1P recovered from cells and media. *B*: ^{32}P-S1P isolated from cells. Values are mean ± SE cpm (n = 3). P values were determined by one-way ANOVA and multiple comparison test.

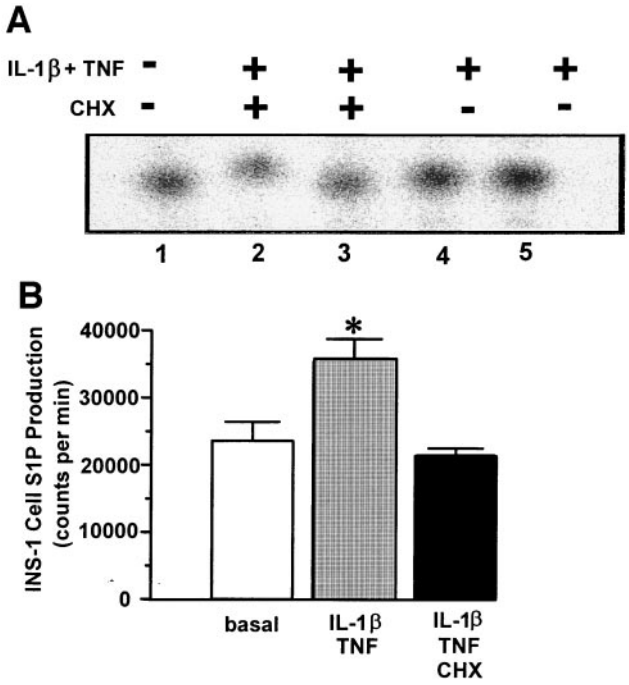

FIG. 8. Endogenous S1P production in INS-1 cells is increased by cytokine treatment and is dependent on new protein synthesis. ^{32}P-prelabeled cells were cultured in the absence (−) or presence (+) of cycloheximide (CHX; 5 μmol/l) for 30 min before an additional 8-h incubation in the absence or presence of IL-1β (2 ng/ml) and TNF-α (20 ng/ml; *lanes 2–5*). S1P was extracted, isolated by TLC, and analyzed by digitized phosphoimage. *A*: Representative phosphoimage from INS-1 cells. *B*: ^{32}P-S1P in cytokine-treated cells in the absence and presence of cycloheximide expressed as mean ± SE cpm (n = 3). Significant differences determined by one-way ANOVA and multiple comparison test. *P < 0.02 vs. basal and cycloheximide-treated samples.

during treatment with IL-1β and TNF-α. The cytokine-induced increase in [^{32}P]S1P levels in cycloheximide-treated cells was reduced by ~40% compared with cells not treated with the inhibitor (Fig. 8). The levels of [^{32}P]S1P in cycloheximide-treated cells were similar to those recovered from cells cultured under basal conditions (Fig. 8).

Regulation of SPHK mRNA. The regulation of SPHK genes during cytokine challenge to β-cells was also investigated. Levels of SPHK1a and SPHK2 mRNAs were normalized to 18S rRNA in INS-1 cells cultured in the absence or presence of IL-1β, TNF-α, and a combination of these cytokines. Culture of INS-1 cells with IL-1β for 1 h significantly increased the ratio of SPHK1a mRNA/18S rRNA by nearly 70% versus basal values (Fig. 9*A*). However, the increase in relative SPHK1a mRNA levels was not sustained when the cells were continuously treated with IL-1β for 8 h (Fig. 9*A*). TNF-α also stimulated an early increase in SPHK1a mRNA within 1 h, but the effect was not apparent after 8 h (Fig. 9*A*). A combination of the cytokines did not raise relative SPHK1a mRNA levels above the levels observed with either cytokine alone after 1 h, and there was no apparent stimulatory effect of the cytokines after 8 h (Fig. 9*A*). In contrast to SPHK1a mRNA, there were no significant changes in the relative levels of INS-1 cell SPHK2 mRNA after stimulation by cytokines (Fig. 9*B*).

Isolated rat pancreatic islets also responded to IL-1β treatment with changes in SPHK mRNA expression.

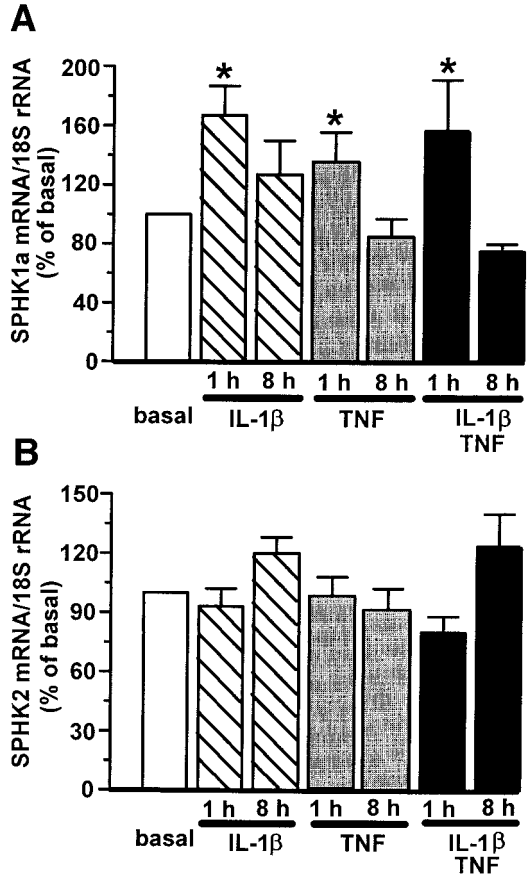

FIG. 9. IL-1β induces SPHK mRNA in INS-1 cells. INS-1 cells were cultured in the absence (basal) or presence of IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for 1 or 8 h. The ratios of SPHK1a (*A*) and SPHK2 (*B*) mRNA levels relative to 18S rRNA were determined by RT-PCR and expressed as percentage of paired basal values. Values are means ± SE for 4–13 independent experiments. *P < 0.01 vs. basal, as determined by one-way ANOVA and multiple comparison test.

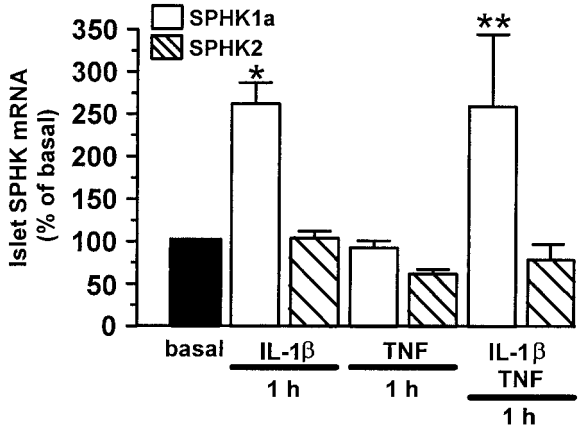

FIG. 10. Islet SPHK isozyme mRNA levels. Islets were cultured in CMRL-1066 media containing 5.5 mmol/l glucose and the absence (basal) or presence of IL-1β (2.5 ng/ml) and/or TNF-α (5–10 ng/ml) for 1 h, as indicated. The ratios of SPHK1a and SPHK2 mRNA levels relative to 18S rRNA were determined by RT-PCR and expressed as percentage of paired basal values. Values are means ± SE for three to four independent experiments. *P < 0.001, **P < 0.03 vs. basal, as determined by one-way ANOVA and multiple comparison test.

Within 1 h, relative SPHK1a mRNA levels in IL-1β–treated islets increased to more than 2.5-fold the basal values; similar results were observed with a combination treatment of IL-1β and TNF-α. However, TNF-α alone failed to elicit any changes in SPHK1a mRNA levels in islets (Fig. 10). Moreover, relative SPHK2 mRNA levels remained unchanged in the face of a 1-h islet challenge by IL-1β or TNF-α or a combination of these cytokines (Fig. 10). After a longer period (8 h) of islet exposure to IL-1β or a combination of IL-1β and TNF-α, the relative levels of SPHK1a mRNA were not significantly different ($P > 0.05$) from basal values (142 ± 15% and 116 ± 40% of basal, respectively); moreover, relative SPHK2 mRNA levels were not significantly different from basal values (131 ± 9% and 94 ± 27% of basal, respectively).

DISCUSSION

S1P is an extracellular receptor ligand and an intracellular signaling molecule. A physiological role for extracellular S1P in rat islets and INS-1 cells mediated by EDG receptors for S1P was recently reported by this laboratory (11). Endogenous S1P has been reported to regulate intracellular Ca^{2+} mobilization and to promote cell growth and survival (9,39,40). SPHK uses sphingosine as a substrate to generate S1P, and activity has been demonstrated in numerous cell types (9,20,23). This study is the first to characterize SPHK expression and activity in rat pancreatic β-cells and demonstrates the intracellular regulation of INS-1 cell and islet SPHK expression, activity, and S1P biosynthesis. Total SPHK activity in INS-1 cell (5.9 ± 1.4 pmol S1P formed · min^{-1} · mg $protein^{-1}$) and islet (3.0 ± 1.5 pmol S1P formed · min^{-1} · mg $protein^{-1}$) cytosolic fractions was similar to the total activity reported for PC12 cell cytosolic fractions (28 ± 4.9 pmol S1P · min^{-1} · mg $protein^{-1}$) (24). However, various tissues express widely different SPHK activities (33). In this study, rat INS-1 cells serve as a homogeneous β-cell model (32) in comparison with isolated rat islets, which are an organ composed of at least three types of endocrine cells and other cell types.

Several cloned SPHKs have been reported for species including human, rat, mouse, yeast, and plant (13). These data illustrate that mRNAs for SPHK1 and SPHK2 isozymes are expressed in rat pancreatic islets and INS-1 insulinoma cells. SPHK used sphingosine as substrate in the production of S1P in these cells. Moreover, the results illustrate that endogenous β-cell SPHK activity is acutely regulated by the cytokines IL-1β and/or TNF-α at concentrations that induce apoptosis. This cytokine-regulated SPHK activation was rapid and sustained and correlated with increased levels of intracellular S1P in INS-1 cells. Cytokines, including IL-1β and TNF-α, are major stress inducers in β-cells and have been implicated in the development of diabetes (27,35–37). Unexpectedly, islet SPHK activation by the combination of IL-1β and TNF-α was not as high as the enzyme activity induced by either cytokine alone within 1 h of treatment, although in INS-1 cells, both cytokines elicited comparable early increases in SPHK activation. However, after 8 h, the SPHK activities of cytokine-stimulated islets and INS-1 cells were comparable. INS-1 cells showed a higher responsivity after 15 min to cytokines than did islets. Although SPHK activity was enriched in the membrane and nuclear subcellular fractions of INS-1 cells, it was the SPHK activity in the cytosolic fraction that was significantly activated by treatment of cells with IL-1β and TNF-α. Whether this can be

attributed to greater instability of the enzyme associated with membranes (34) is not known.

SPHK activation appeared to occur in at least two phases: an early phase (~15 min) that is expected to be largely independent of new protein synthesis, and a late phase that is dependent on new protein synthesis. After 8 h, cycloheximide blocked most, if not all, of the cytokine-induced increase in S1P levels, suggesting that activation of the SPHK pathway involved activation of endogenous enzyme as well as induction of new enzyme. Kinases and signaling proteins might contribute to early activation of SPHK. SPHK is a largely cytosolic enzyme (39) that has been postulated to migrate to membranes where it can phosphorylate sphingosine (41). PKC-activating phorbol esters have been reported to activate SPHK (19). In preliminary studies, phorbol 12-myristate 13-acetate activated INS-1 cell cytosolic SPHK activity (L.D.M., unpublished data). Putative phosphorylation sites in SPHK may regulate activity. Phorbol esters can induce phosphorylation of SPHK1 (38), presumably via PKC and a mitogen-activated serine/threonine protein kinase (42). Interestingly, IL-1β in islets acutely increases PKC activation (43), and TNF-α can potentiate IL-1β–induced PKC activity (44). Recently, phorbol ester–induced HEK-293 cell SPHK activation was reported to involve translocation of SPHK to the plasma membrane (38). Ca^{2+}/calmodulin may also activate SPHK (40). Paralleling an increase in PKC activation by IL-1β is an increase in intracellular Ca^{2+} (45). Thus, the activation of islet SPHK may be modulated by Ca^{2+} and PKC. In addition, TNF-α receptor–associated factor-2, nuclear coactivator 62-kDa/Ski-interacting protein, and a novel SPHK-1-binding protein, RPK118, bind to SPHK and may regulate SPHK activation (46–48).

A role for SPHK and the phosphorylated lipid product, S1P, in β-cells may be related to S1P having actions on apoptosis and cell growth opposing those of ceramide and sphingosine (14,39). Exposure of β-cells to SPHK-activating agents converts sphingosine to S1P, which may reduce the apoptotic potential. In addition, in some cells, S1P can regulate intracellular Ca^{2+} mobilization and promote cell growth and survival (9,39,40). On the other hand, it has been reported that SPHK2 promotes apoptosis (25). Thus, SPHK2 may be a heretofore unrecognized modulator of cytokine-induced apoptosis in β-cells.

IL-1β and TNF-α, alone or in combination, also stimulated an early increase in SPHK1a mRNA expression in INS-1 cells, whereas in rat islets, IL-1β but not TNF-α stimulated an early (1 h) increase in SPHK1a mRNA expression. The differences between islets and INS-1 cells could be related to the interaction of cytokine and glucose stimulation (11 mmol/l) in the INS-1 cell cultures versus 5.5 mmol/l glucose in the islet cultures or to inherent differences between transformed and primary cells. In INS-1 cells or islets, cytokine effects on SPHK1a mRNA levels were not sustained over 8 h, suggesting that mRNA stability and/or transcription may be affected.

Surprisingly, cytokines failed to affect the expression of SPHK2 mRNA in islets or INS-1 cells. These data are in contrast to the cytokine effects on SPHK activity levels in INS-1 cells, where it appeared that SPHK2 was the predominant isoform and that cytokines elicited maximal stimulation that was not further affected by high salt concentration. In contrast, SPHK activity in the presence of Triton X-100 was low in cytokine-treated INS-1 cell extracts. The residual SPHK activity observed in the presence of Triton X-100 may be SPHK1; however, there was no cytokine regulation of this activity over a 24-h period. The tight regulation of SPHK activity in the presence of cycloheximide suggests that new protein synthesis does account for long-term SPHK activation. If this late chronic S1P-synthesizing activity is accounted for by SPHK2, then increased translation of SPHK2 or enzyme activation must explain the results. There is no ready explanation for why SPHK1a isoform mRNA appears to be highly regulated in these cells but is not paralleled by a change in enzyme activity. It is possible that SPHK1 was exported from the cells (49) or is unstable or that levels of the enzyme defy biochemical detection.

The subcellular distribution of SPHK2 in INS-1 cells and islets in nuclei and cytosol is typical (27). Whereas SPHK1 was expected to be localized to the cytosol, little, if any, SPHK1 activity was observed in the presence of Triton X-100. This is in contrast to the Triton X-100–induced activation of a recombinant murine SPHK1a transfected into INS-1 cells. Localization of SPHK2 activity and S1P production in the β-cell nucleus may modulate changes in gene transcription or even mitogenesis (34). A role for the regulated expression of SPHK1a mRNA, however, remains to be determined.

In summary, SPHK is active in isolated islets and the β-cell line INS-1, and activity is responsive to cytokines, resulting in enhanced S1P levels. The failure to recover S1P in culture media suggests that islet S1P production would not contribute in a meaningful way to blood levels of S1P or EDG receptor stimulation by autocrine mechanisms in vivo even in the islet microenvironment. The levels of S1P in plasma can be in the 400- to 500-nmol/l range (6). Thus, S1P produced in β-cells is likely to have an intracellular site(s) of action. The rapid response of SPHK to cytokine treatment suggests that it potentially either modulates or offers protection against stress responses induced by cytokines.

ACKNOWLEDGMENTS

S.G.L. was supported by a grant from the Juvenile Diabetes Research Foundation International (1-2002-613). L.D.M. was supported by fellowships from the Endocrine Fellows Foundation and the Juvenile Diabetes Research Foundation International.

We thank Dr. Sarah Spiegel for her generous provision of recombinant mouse SPHK1a plasmid.

REFERENCES

1. Zhang H, Desai NN, Olivera A, Seki T, Brooker G, Spiegel S: Sphingosine-1-phosphate, a novel lipid involved in cellular proliferation. *J Cell Biol* 114:155–167, 1991
2. Hannun YA: Functions of ceramide in coordinating cellular responses to stress. *Science* 274:1855–1859, 1996
3. Hannun YA, Obeid LM: Ceramide and the eukaryotic stress response. *Biochem Soc Trans* 25:1171–1175, 1997
4. Spiegel S, Cuvillier O, Edsall LC, Kohama T, Menzeleev R, Olah Z, Olivera A, Pirianov G, Thomas DM, Tu Z, Van Brocklyn JR, Wang F: Sphingosine-1-phosphate in cell growth and cell death. *Ann N Y Acad Sci* 845:11–18, 1998
5. Pyne S, Pyne NJ: Sphingosine-1-phosphate signaling in mammalian cells. *Biochem J* 349:385–402, 2000

6. Spiegel S, Milstien S: Sphingosine-1-phosphate: signaling inside and out. *FEBS Lett* 476:55–57, 2000
7. Spiegel S, Milstien S: Sphingosine-1-phosphate, a key cell signaling molecule. *J Biol Chem* 277:25851–25854, 2002
8. Van Brocklyn JR, Lee M-J, Menzeleev R, Olivera A, Edsall L, Cuvillier O, Thomas DM, Coopman PJP, Thangada S, Liu CH, Hla T, Spiegel S: Dual actions of sphingosine-1-phosphate: extracellular through Gi-coupled receptor EDG-1 and intracellular to regulate proliferation and survival. *J Biol Chem* 142:229–242, 1998
9. 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* 147:545–558, 1999
10. Goetzl EF, Lee H, Dolezalova H, Kalli KR, Conover CA, Hu Y-L, Azuma T, Stossel TP, Karliner JS, Jaffe RB: Mechanisms of lysolipid phosphate effects on cellular survival and proliferation. *Ann N Y Acad Sci* 905:177–187, 2000
11. Laychock SG, Tian Y, Sessanna S: Endothelial differentiation gene (EDG) receptors in pancreatic islets and INS-1 cells. *Diabetes* 52:1986–1993, 2003
12. Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstien S, Kohama T, Spiegel S: Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem* 275:19513–19520, 2000
13. Liu H, Chakravarty D, Maceyka M, Milstein S, Spiegel S: Sphingosine kinases: a novel family of lipid kinases. *Prog Nucleic Acid Res Mol Biol* 71:493–511, 2002
14. Kohama T, Olivera A, Edsall L, Nagiec MM, Dickson R, Spiegel S: Molecular cloning and functional characterization of murine sphingosine kinase. *J Biol Chem* 273:23722–23728, 1998
15. Pitson SM, Moretti PAB, Zebol JR, Xia P, Gamble JR, Vadas MA, D'Andrea RJ, Wattenberg BW: Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation. *J Biol Chem* 275:33945–33950, 2000
16. Meyer zu Heringdorf D, Lass H, Alemany R, Laser KT, Neumann E, Zhang C, Schmidt M, Rauen U, Jakobs KH, van Koppen CJ: Sphingosine kinase-mediated Ca^{2+} signaling by G-protein-coupled receptors. *EMBO J* 17: 2830–2837, 1998
17. Kimura K, Bowen C, Spiegel S, Gelmann EP: Tumor necrosis factor-alpha sensitizes prostate cancer cells to gamma-irradiation-induced apoptosis. *Cancer Res* 59:1606–1614, 1999
18. Edsall LC, Pirianov GG, Spiegel S: Involvement of spingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. *J Neurosci* 17:6952–6960, 1997
19. Mazurek N, Megidish T, Hakomori S-I, Igarashi Y: Regulatory effect of phorbol esters on sphingosine kinase in BALB/C 3T3 fibroblasts (variant A31): demonstration of cell type-specific response—a preliminary note. *Biochem Biophys Res Commun* 198:1–9, 1994
20. Buehrer BM, Bardes ES, Bell RM: Protein kinase C-dependent regulation of human erythroleukemia (HEL) cell sphingosine kinase activity. *Biochim Biophys Acta* 1303:233–242, 1996
21. Kleuser B, Cuvillier O, Spiegel S: 1α,25-dihydroxyvitamin D_3 inhibits programmed cell death in HL-60 cells by activation of sphingosine kinase. *Cancer Res* 58:1817–1824, 1998
22. Machwate M, Rodan SB, Rodan GA, Harada SI: Sphingosine kinase mediates cyclic AMP suppression of apoptosis in rat periosteal cells. *Mol Pharmacol* 54:70–77, 1998
23. Xia P, Wang L, Gamble JR, Vadas MA: Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. *J Biol Chem* 274:34499–34505, 1999
24. Edsall LC, Cuvillier O, Twitty S, Spiegel S, Milstien S: Sphingosine kinase expression regulates apoptosis and caspase activation in PC12 cells. *J Neurochem* 76:1573–1584, 2001
25. Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H, Payne SG, Bektas M, Ishii I, Chun J, Milstien S, Spiegel S: Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem* 278:40330–40336, 2003
26. Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S, Nakamura S: Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. *J Biol Chem* 278:46832–46839, 2003
27. 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* 139:4077–4082, 1987
28. Laychock SG, Bauer AL: Epiandrosterone and dehydroepiandrosterone affect glucose oxidation and interleukin-1β effects in pancreatic islets. *Endocrinology* 137:3375–3385, 1996
29. Corbett JA, Sweetland MA, Wang JL, Lancaster JR Jr, McDaniel ML: Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc Natl Acad Sci U S A* 90:1731–1735, 1993
30. Mandrup-Poulsen T, Bendtzen K, Nerup J, Dinarello CA, Svenson M, Nielsen JH: Affinity-purified human interleukin 1 is cytotoxic to isolated islets of Langerhans. *Diabetologia* 29:63–67, 1986
31. Xia M, Laychock SG: Insulin secretion, myo-inositol transport and Na+K+-ATPase activity in glucose-desensitized rat islets. *Diabetes* 42: 1392–1400, 1993
32. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin secreting cell lines. *Endocrinology* 130:167–178, 1992
33. Olivera A, Kohama T, Tu Z, Milstien S, Spiegel S: Purification and characterization of rat kidney sphingosine kinase. *J Biol Chem* 273:12576–12583, 1998
34. Kleuser B, Maceyka M, Milstien S, Spiegel S: Stimulation of nuclear sphingosine kinase activity by platelet-derived growth factor. *FEBS Lett* 503:85–90, 2001
35. 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-κ-dependent genes in primary rat pancreatic β-cells. *J Biol Chem* 276:48879–48886, 2001
36. Hoorens A, Stange G, Pavlovic D, Pipeleers D: Distinction between interleukin-1–induced necrosis and apoptosis of islet cells. *Diabetes* 50: 551–557, 2001
37. Chang I, Kim S, Kim JY, Cho N, Kim YH, Kim HS, Lee MK, Kim KW, Lee MS: Nuclear factor κB protects pancreatic β-cells from tumor necrosis factor-α–mediated apoptosis. *Diabetes* 52:1169–1175, 2003
38. Johnson KR, Becker KP, Facchinetti MM, Hannun YA, Obeid LM: PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. *J Biol Chem* 277:35257–35262, 2002
39. Olivera A, Spiegel S: Sphingosine kinase: a mediator of vital cellular functions. *Prostaglandins Lipid Med* 64:123–134, 2001
40. Young KW, Nahorski SR: Sphingosine 1-phosphate: a Ca^{2+} release mediator in the balance. *Cell Calcium* 32:335–341, 2002
41. Young KW, Willets JM, Parkinson MJ, Bartlett P, Spiegel S, Nahorski SR, Challiss RA: Ca^{2+}/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane relocation but not activation. *Cell Calcium* 33:119–128, 2003
42. Pitson SM, Moretti PAB, Zebol JR, Lynn HE, Xia P, Vadas MA, Wattenberg BW: Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J* 22:5491–5500, 2003
43. Eizirik DL, Sandler S, Welsh N, Juntii-Berggren L, Berggren PO: Interleukin-1β-induced stimulation of insulin release in mouse pancreatic islets is related to diacylglycerol production and protein kinase C activation. *Mol Cell Endocrinol* 111:159–165, 1995
44. Messmer UK, Brune B: Modulation of inducible nitric oxide synthase in RINm5F cells. *Cell Signal* 6:17–24, 1994
45. Borg LA, Eizirik DL: Short-term exposure of rat pancreatic islets to human interleukin-1β increases cellular uptake of calcium. *Immunol Lett* 26:253–258, 1990
46. Xia P, Wang L, Moretti PAB, Albanese N, Chai F, Pitson SM, D'Andrea RJ, Gamble JR, Vadas MA: Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-α signaling. *J Biol Chem* 277:7996–8003, 2002
47. Lacaná E, Maceyka M, Milstien S, Spiegel S: Cloning and characterization of a protein kinase A anchoring protein (AKAP)-related protein that interacts and regulates sphingosine kinase1 activity. *J Biol Chem* 277: 32947–32953, 2002
48. Hayashi S, Okada T, Iagarashi N, Fujita T, Jahangeer S, Nakamura S: Identification and characterization of RPK118, a novel sphingosine kinase-1-binding protein. *J Biol Chem* 277:33319–33324, 2002
49. Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae S-S, Stefansson S, Liau G, Hla T: Extracellular export of sphingosine kinase-1 enzyme: sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. *J Biol Chem* 277:6667–6675, 2002