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Sphingosine 1-Phosphate Affects Cytokine-Induced Apoptosis in Rat Pancreatic Islet β-Cells

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Cytokines mediate pancreatic islet β-cell apoptosis and necrosis, leading to loss of insulin secretory capacity and type 1 diabetes mellitus. The cytokines, IL-1β and interferon-γ, induced terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining of rat islet cells within 48 h by about 25–30%, indicative of apoptosis and/or necrosis. Sphingosine 1-phosphate (S1P) at nanomolar concentrations significantly reduced islet cell cytokine-induced TUNEL staining. Similar effects were observed in INS-1 cells. The dihydro analog of S1P also reduced the percentage of TUNEL stained islet and INS-1 cells, whereas the S1P receptor antagonist BML-241 blocked the protective effects. Pertussis toxin did not affect the S1P protective response. In the presence of a phospholipase C antagonist, U73122, there was significant inhibition of the S1P protective effects against apoptosis/necrosis. S1P stimulated INS-1 cell protein kinase C activity. Carbamylcholine chloride acting through muscarinic receptors also inhibited cytokine-induced TUNEL staining in pancreatic islet cells. S1P and/or dihydro-S1P also antagonized cytokine-induced increases in cytochrome c release from mitochondria and caspase-3 activity in INS-1 cells, which are indicative of cell apoptosis *vs.* necrosis. S1P failed to affect nitric oxide synthase activity after 48 h. Thus, the evidence suggests that S1P acting on S1P receptors coupled to G_q mediates protective effects on islet β-cells against cytokine-induced apoptosis. (*Endocrinology* 147: 4705–4712, 2006)

THE β-CELLS OF the pancreatic islet of Langerhans secrete insulin in response to glucose, amino acids, hormones, and neurotransmitters (1). However, in both types 1 and 2 diabetes mellitus, there are stress signaling pathways that contribute to loss of β-cell secretory responsiveness and induction of β-cell apoptosis and death (2). Apoptosis mainly accounts for human islet β-cell death in early type 1 diabetes in response to cytokines (3, 4). The cytokines IL-1β and TNFα induce not only gene expression but also necrosis and apoptosis (5). Apoptosis also plays a role in type 2 diabetes, in which it may be mediated/initiated by free fatty acids, glucose, sulfonylurea, amylin, MAPKs, and stress-activated protein kinase (SAPK)/c-Jun NH_2-terminal kinase (JNK) and increases in ceramide (2, 6, 7). Long-chain fatty acids implicated in type 2 diabetes contribute to increased ceramide levels in islets (8).

A unique bioactive phospholipid, sphingosine-1 phosphate (S1P), is a metabolite of sphingosine and has been described as a potent stimulus for DNA synthesis, Ca^{2+} mobilization, and MAPK pathway activation (9, 10). S1P can act both as an intracellular second messenger and a receptor ligand. The S1P (or endothelial differentiation gene (EDG)] receptor class includes a family of receptor isoforms (S1P-1, -2, -3, -4, and -5) that bind S1P with high affinity and specificity (9, 10, 11). This laboratory identified four S1P receptor isoforms ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$) in rat and mouse islets and INS-1 cells (12). $S1P_3$ has also been identified in HIT-T 15 clonal β-cells (13). Another family of EDG receptors (EDG-2, -4, and -7) binds lysophosphatidic acid.

S1P receptors are GTP-binding (G) protein-coupled cell surface receptors that regulate diverse signal transduction pathways and elicit pleiotropic responses unique to the cell type and relative expression of S1P receptors. $S1P_1$ is an immediate-early gene (14) that inhibits adenylyl cyclase (11) and activates ERK (15) through G_i. $S1P_2$ and $S1P_3$ are coupled predominantly to G_q and activate phospholipase C (PLC) to induce Ca^{2+} mobilization through the production of inositol 1,4,5-trisphosphate (16, 17), and induce activation of ERK, SAPK/JNK, and p38 MAPK (18).

Besides receptor ligand binding, S1P also has intracellular sites of action. S1P mobilizes intracellular Ca^{2+} from internal stores independent of inositol 1,4,5-trisphosphate generation and activates ERK but inhibits SAPK/JNK. The latter S1P activities contribute to promotion of cell proliferation and suppression of apoptosis, respectively (10). Ceramide and S1P, products of sphingomyelin metabolism, appear to represent opposing signaling cascades, the former signifying cell growth arrest and the latter proliferation and survival. In T lymphocytes, stress increases ceramide and sphingosine leading to apoptosis, whereas survival factors increase S1P levels (19). In β-TC3 insulinoma cells, ceramide analogs mimicked the cytotoxic effects of cytokines (20), suggesting a role for ceramide in β-cell apoptotic events.

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Abbreviations: CCh, Carbamylcholine chloride; EDG, endothelial differentiation gene; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; G, GTP-binding; HDL, high-density lipoprotein; IFN, interferon; JNK, c-Jun NH_2-terminal kinase; MOPS, 3(*N*-morholino)propanesulfonic acid; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; SAPK, stress-activated protein kinase; S1P, sphingosine-1 phosphate; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated deoxyuridine triphosphate nick end labeling.

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In the present study, the potential for S1P to promote β-cell survival in the face of cytokine challenge is investigated.

Materials and Methods

Materials

D-*erythro*-S1P and D-*erythro*-dihydro-S1P were from Biomol International, LP (Plymouth Meeting, PA). Collagenase was obtained from Crescent Chemical Co. (Islandia, NY). CMRL-1066 and RPMI 1640 culture media, *Taq* DNA polymerase, random hexamer, Superscript II RNase H^-, glycogen, RNaseOUT, DNase 1 amplification grade, deoxynucleotide triphosphates, and TRIzol were from Invitrogen/Life Technologies (Grand Island, NY). QuantumRNA Classic 18S standards (488 bp) were from Ambion Inc. (Austin, TX). Fetal bovine serum (FBS) was from Atlanta Biologicals, Inc. (Norcross, GA). rh-IL-1β, rr-interferon-γ (IFN), rr-TNFα, and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) FlowTACS apoptosis detection kit were from R&D Systems Inc. (Minneapolis, MN). BML-241 was from Biomol International LP. Protein assay reagent was from Bio-Rad Laboratories (Hercules, CA). 3-Isobutyl-1-methylxanthine, BSA fraction V (insulin free and fatty acid free), phorbol 12-myristate 13-acetate (PMA), carbamylcholine chloride (CCh), phycoerythrin-streptavidin conjugate, and protease inhibitor cocktail were obtained from Sigma Chemical Co. (St. Louis, MO). Cytochrome c antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). CaspACE fluorometric caspase-3 assay system was from Promega Corp. (Madison, WI). Protein kinase C (PKC) assay kit (Upstate, Lake Placid, NY). U73122 was a gift from the Upjohn Laboratories (Kalamazoo, MI). INS-1e cells were a gift from Dr. Claes Wollheim (University Medical Center, Geneva, Switzerland). All other chemicals were reagent grade.

Isolation of rat islets and culture of cells and tissue

Pancreatic islets were isolated from pancreata of adult male Sprague Dawley rats using collagenase, essentially as described previously (21). All animal procedures were approved by the institutional animal care and use committee. Freshly isolated islets (fresh) were either used immediately or cultured in CMRL-1066 for up to 2 d in the presence or absence of various stimuli or inhibitors as described in the text. CMRL-1066 medium contained 5.5 mM glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and the presence of 10% FBS or 0.1% fatty acid-free BSA, as indicated in the text. Culture conditions were 5% CO_2-95% air and 37 C.

INS-1 cells were cultured in RPMI 1640, as described previously (22), in the presence of 5% FBS or 0.1% fatty acid-free BSA, as indicated in the text.

TUNEL analysis

In preparation for islet cell TUNEL analysis, approximately 200 islets per treatment group were cultured overnight in CMRL-1066 containing 0.1% fatty acid-free BSA in the absence of serum to remove serum sources of S1P. Then S1P or dihydro-S1P or CCh was added to islet cultures for 4.5–5 h before the addition of cytokines, and culture was continued for 48 h after initiation of cytokine treatment. INS-1 cells in RPMI 1640 were treated similarly to islets. The FlowTACS apoptosis detection kit allows visual detection of DNA fragmentation with biotinylated nucleotides incorporated into the free 3′-hydroxyl residues of the DNA fragments. Streptavidin-conjugated phycoerythrin binds the biotinylated DNA fragments and can be detected by flow cytometry. However, necrotic cells can also contain DNA fragments that would be detected by this method. Thus, in this study apoptosis/necrosis are not distinguished by this method. TUNEL analysis was performed on islet cells after a 48-h culture in the absence or presence of cytokines. The islets (100–120 islets/sample) were washed with PBS, followed by suspension in versene (1 mM EDTA in PBS) for 8 min. The islets were briefly centrifuged and resuspended in 1 mM trypsin in Hank's buffered saline solution, and incubated at 37 C for 4 min, followed by repeated aspiration (approximately 40 times) until the islets were dissociated into single cells. Then the cells were washed and incubated for 1 h at 37 C in CMRL-1066 medium containing 5% FBS, followed by washing twice with PBS. The cells were then processed for TUNEL staining according to the manufacturer's specifications for the TUNEL FlowTACS apoptosis detection kit, including fixation in formaldehyde, permeabilization, and treatment with TdT labeling buffer, deoxyuridine triphosphate-biotin, manganese, and TdT enzyme. After reaction with phycoerythrin-streptavidin conjugate, the cells were resuspended in PBS, and TUNEL staining was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) with at least 30,000 cells counted per sample. Using the histogram subtraction method, the number of untreated control cells exhibiting basal TUNEL staining and autofluorescence was subtracted from all other paired treatment groups in each experiment. The data are expressed as percent apoptotic cells relative to the total cells counted as determined using CellQuest software (BD Biosciences). INS-1 cells were prepared similarly to islet cells for TUNEL staining.

PKC assay

INS-1 cell PKC activity was determined according to the manufacturer's instructions for the PKC assay kit. INS-1 cells were cultured for 10 min in the absence or presence of PMA or S1P. The confluent cell monolayer were washed twice in cold PBS, scrapped into 100 μl 3[*N*-morholino]propanesulfonic acid (MOPS) homogenization buffer [20 nM MOPS (pH 7.2), 1 mM sodium orthovanadate, 1 mM $CaCl_2$, and protease inhibitor cocktail], and then sonicated for 5 sec. A 20-μl aliquot was removed from whole lysate for assay. The remaining cell lysate was centrifuged for 1 h at 100,000 × *g* at 4 C. The resulting supernatant was saved, and the pellet (particulate) fraction was resuspended in 50 μl MOPS homogenization buffer and sonicated 20 sec. Protein levels in each fraction were determined by Bio-Rad protein assay. The PKC reactions were carried out using 25 μg protein in the absence and the presence of substrate peptide (QKRPSQRSKYL) according to the kit instructions. PKC activity was determined as picomole of ^{32}P incorporated into substrate peptide per minute per milligram protein. Values for treated INS-1 cell samples were normalized against control values for percent change in PKC activity.

Caspase-3 assay

INS-1 cells were washed once with RPMI 1640 containing 5% serum and then twice more in cold PBS. The cells were centrifuged at 1000 × *g* at 4 C and resuspended in 50 μl hypotonic cell lysis buffer consisting of 25 mM HEPES, 5 mM magnesium chloride, 5 mM EDTA, 5 mM dithiothreitol, and 10 μl/ml protease inhibitor cocktail. The cells were frozen and thawed one time and then sonicated for 4 sec. The homogenate was then centrifuged at 18,000 × *g* for 20 min at 4 C, and the supernatant was used to assay caspase-3 activity using Promega CaspACE fluorometric assay system.

Cytochrome c analysis

INS-1 cells were treated with or without S1P (400 nM) during a 5-h preincubation, which was followed by the addition of IL-1β (1 ng/ml), TNF (10ng/ml), and IFN (10 ng/ml) with continued culture for 1 d. The cells were then washed twice with cold PBS and scraped into 100 μl of cold mitochondrial isolation buffer (0.25 M sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM EDTA, 1.5 mM EGTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 mg/ml protease inhibitor cocktail) and then incubated on ice for 30 min. The cells were lysed by 15 passes of a type B dounce homogenizer and then centrifuged at 1500 × *g* for 10 min. The resulting supernatant was centrifuged at 22,000 × *g* for 15 min to remove intact mitochondria, and the supernatant cytosolic fraction was used for Western blotting of cytochrome c. The relative amounts of cytochrome c in the cytosolic fractions were determined by densitometric analysis, and results are expressed as percent of paired control samples on the same blot.

Nitric oxide synthase assay

Nitric oxide synthase activity was estimated by the accumulation of nitrite in INS-1 cell cultures after 24 or 48 h, essentially as described previously (23). Nitrite values were normalized to cellular total protein content.

Statistical analysis

Significant differences between samples were determined by Student's *t* test (paired, two tailed) or one-way ANOVA with Student/Newman-Keuls multiple comparison test. $P \leq 0.05$ were accepted as significant.

Results

TUNEL staining in pancreatic islets and INS-1 cells

TUNEL staining of rat islet cells was used to determine the percentage of cells that were in the process of undergoing, or had undergone, apoptosis and/or necrosis. Rat pancreatic islets were cultured for 24 h in the absence of serum to deprive them of exogenous S1P. A cytokine cocktail consisting of IL-1β and IFN was added to islets in the absence or presence of S1P. After 48 h, the treated islet cells were analyzed by fluorescence-activated cell sorter (FACS) for TUNEL staining *vs.* untreated islet cells to determine the proportion of cells undergoing apoptosis and/or necrosis. The combination of IL-1β and IFN produced an increase in TUNEL-stained cells that was approximately 25–30% of total cells (Fig. 1, A and C). As shown in Fig. 1A, the cytokines increased TUNEL staining *vs.* basal phycoerythrin staining. Low concentrations of S1P in the 100–400 nmol range proved to be protective of islet cells against apoptosis/necrosis induced by the cytokines (Fig. 1, B and C). TUNEL staining was shifted toward basal values when S1P was present during the cytokine challenge (Fig. 1B). However, a higher concentration of S1P (1 μM) was less effective than 400 nM S1P at protecting the islet cells (Fig. 1C). S1P alone at 200 or 400 nM had no significant effect on islet cell TUNEL staining, compared with control cells in the absence of cytokine stimulation (Fig. 1C). The histogram subtraction method subtracts basal background and apoptotic cell staining from paired treatment groups to quantitate changes in TUNEL staining. However, to quantitate a basal level of apoptosis in islet cells, fluorescence in TdT-stained cells was compared with fluorescent cells lacking enzyme treatment. Islets showed a basal level of 15.6 ± 1.2% apoptotic cells.

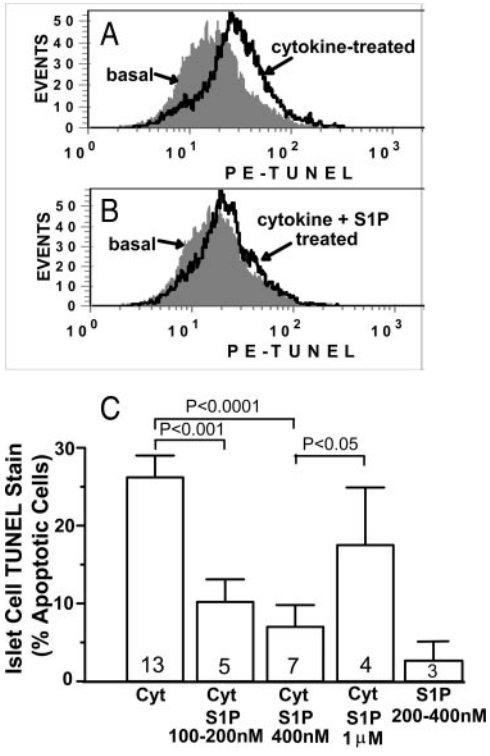

FIG. 1. TUNEL staining of islet cells. A and B, Representative FACS analysis of TUNEL-stained islet cells. A and B, Histogram subtraction provides the number of apoptotic cells above basal for each treatment group. The *clear area* shown between basal phycoerythrin (PE) TUNEL staining (*gray shaded area*) and the cytokine-treated cells (A) or cytokine plus S1P-treated cells (B) is the difference in the number of apoptotic cells (events) due to the treatments. C, Islets were treated with S1P (at the concentrations shown) in the absence or presence of IL-1β (5 ng/ml) and IFN (10 ng/ml) (Cyt) for 48 h, as indicated. TUNEL staining was determined for dissociated islet cells by FACS analysis and expressed as mean ± SE percent of total cells sorted (30,000 cells). The number of independent experiments is shown at the base of each bar. *P* values were determined by one-way ANOVA and multiple comparison test.

To differentiate between the extracellular and intracellular actions of S1P on islet cells, the islets were cultured in the presence of dihydro-S1P, a selective S1P receptor agonist that lacks the double bond at C-4 position of S1P and does not mimic S1P intracellular responses (24). Dihydro-S1P (400 nM) protected the islet cells from apoptosis/necrosis to a similar extent as S1P at an equimolar concentration (Fig. 2). Dihydro-S1P alone did not significantly affect islet cell TUNEL staining (Fig. 2). The mediation of dihydro-S1P effects by cell surface receptors was determined with the EDG receptor antagonist BML-241 (25). In the presence of BML-241, dihydro-S1P inhibition of islet cell cytokine-induced apoptosis/necrosis was prevented and the percentage of TUNEL-stained cells was similar to cytokine-treated cells (Fig. 3). The presence of BML-241 with the cytokines did not alter the percentage of cells undergoing apoptosis/necrosis, compared with cytokine treatment alone (Fig. 3). BML-241 alone had no effect on the occurrence of apoptosis/necrosis (3% apoptotic cells) in islet cells (data not shown).

Evidence for the apoptotic protective effects of S1P in a homogeneous population of β-cells was investigated using

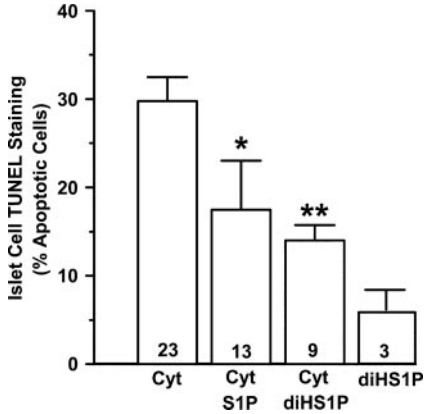

FIG. 2. Dihydro-S1P protects islet cells from cytokine-induced apoptosis/necrosis. Islets were treated with dihydro-S1P (diHS1P) (400 nM) or S1P (400 nM) in the presence or absence of IL-1β (5 ng/ml) and IFN (10 ng/ml) (Cyt) treatment for 48 h, as indicated. Values for the percent of TUNEL-stained cells are means ± SE for the number of independent experiments shown at the base of each bar. *, $P < 0.01$ and **, $P < 0.001$ *vs.* Cyt, as determined by one-way ANOVA and multiple comparison test.

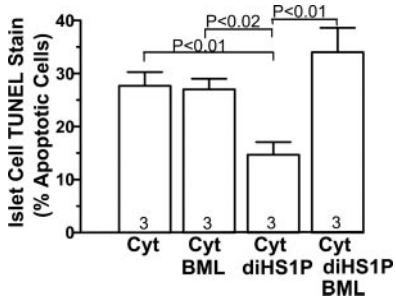

FIG. 3. BML-241 S1P receptor antagonism of islet cell TUNEL staining. Islets were treated with BML-241 (BML) (10 μM) and/or dihyro-S1P (diHS1P) (400 nM) in the presence of IL-1β (2.5 ng/ml) and IFN (5 ng/ml) (Cyt) for 48 h. Values for the percent of TUNEL stained cells are means ± SE for the number of independent experiments shown at the base of each bar. *P* values were determined by one-way ANOVA and multiple comparison test.

INS-1 cells treated with a combination of cytokines (IL-1β, IFN, and TNF). INS-1 cells responded to the cytokine cocktail with an increase in TUNEL staining of about 40–50% (Fig. 4). In comparison with islets, the combination of the three cytokines gave more reproducible induction of apoptosis/necrosis in INS-1 cells and was used in all subsequent INS-1 cell TUNEL experiments. In the presence of S1P, INS-1 cells showed significantly less TUNEL staining than cells treated with cytokines alone (Fig. 4). Dihydro-S1P (0.2 μM to 1 μM) also reduced the number of TUNEL-stained INS-1 cells with cytokine treatment (Fig. 4). Basal INS-1 cell apoptosis determined by comparison of fluorescence in TdT-stained cells *vs.* fluorescent cells lacking enzyme treatment showed a basal 3.8 ± 2.2% apoptosis in INS-1 cells.

To determine whether S1P receptors coupled to G_i mediated the S1P protective response in islet cells, islets were treated with pertussis toxin to inhibit G_i activity. In the presence of IL-1β (2.5 ng/ml) and IFN (5 ng/ml) 34 ± 2% of cells were TUNEL stained, and the addition of S1P (400 nM) reduced TUNEL staining to 13 ± 2% of total cells ($P < 0.05$). When paired islets were treated with IL-1β (2.5 ng/ml), IFN (5 ng/ml), S1P (400 nM), and pertussis toxin (100–200 ng/ml), only 16 ± 2% of cells were TUNEL stained ($P < 0.05$ *vs.* cytokine-treated cells), showing a protective response similar to that afforded by S1P in the absence of pertussis toxin. Pertussis toxin (200 ng/ml) alone showed a small effect (7%) on TUNEL staining in islet cells. A preliminary study also showed that clonidine (1 μM), an α_2-adrenergic receptor agonist that acts through G_i, did not protect islets against the 2-d cytokine-induced apoptosis/necrosis (data not shown).

Because G_q coupling also mediates the signal transduction response to certain S1P receptors, the G_q-mediated PLC-stimulated pathway was inhibited with U73122 to determine effects on islet cell apoptosis/necrosis. Treatment of islets with S1P reduced the percentage of TUNEL-stained cytokine-treated cells by approximately 50%, and the presence of U73122 caused a significant antagonism of the S1P protective effect against cytokine-induced apoptosis/necrosis (Fig. 5). In contrast, when U73122 was combined with cytokine stimulation, the percentage of TUNEL stained cells was not different from that observed with cytokine challenge alone, and U73122 alone did not significantly alter the number of TUNEL-stained cells (Fig. 5).

In comparison with S1P receptors, muscarinic receptors are also coupled to G_q and mediate PLC stimulation. When islets were stimulated with CCh, a muscarinic receptor agonist, there was significant inhibition of the cytokine-induced TUNEL staining (Fig. 6). CCh alone induced a small elevation in TUNEL-stained islet cells that was not different from cytokines together with CCh (Fig. 6).

PKC activity

The response of PKC to S1P was determined in INS-1 cells. Addition of S1P to INS-1 cells resulted in an approximately 77% increase in PKC activity in whole-cell homogenates, and a similar increase was observed in the particulate subcellular fraction (Table 1). In comparison, PMA stimulated INS-1 cell whole homogenate and particulate PKC activity approximately 4-fold (Table 1).

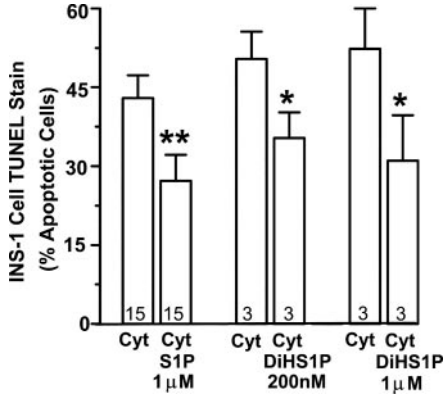

FIG. 4. S1P and dihydro-S1P (diHS1P) effects on INS-1 cell TUNEL stain. INS-1 cells were treated with S1P or diHS1P at the concentrations shown and a combination of IL-1β (1 ng/ml), IFN (10 ng/ml), and TNF (10 ng/ml) (Cyt), as indicated, for 48 h. Percentage of INS-1 cell TUNEL staining is expressed as means ± SE for the number of independent experiments shown at the base of each bar. **, $P < 0.01$ and *, $P < 0.05$ *vs.* paired Cyt values were determined by paired two-tailed Student's *t* test.

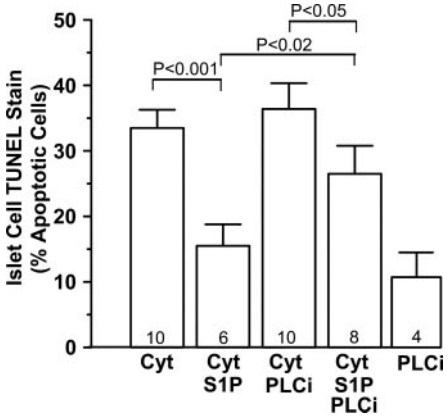

FIG. 5. Effect of a PLC inhibitor on islet cell TUNEL staining. Islets were treated with U73122 (PLCi) (1 μM) and/or S1P (400 nM), in the absence or presence of IL-1β (2.5 ng/ml) and IFN (5 ng/ml) (Cyt), for 48 h, as indicated. Values for the percentage of TUNEL-stained cells are means ± SE for the number of independent experiments shown at the base of each bar. *P* values were determined by one-way ANOVA and multiple comparison test.

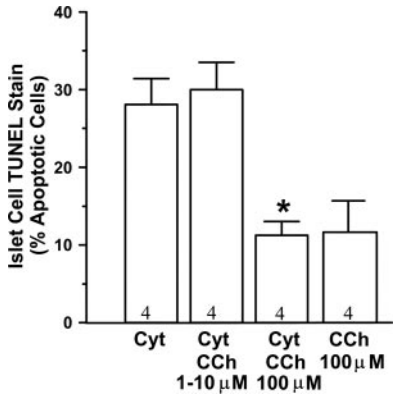

FIG. 6. Effect of carbachol on islet cell TUNEL stain. Islets were treated with carbachol (at the concentrations shown) in the absence or presence of IL-1β (2.5 ng/ml) and IFN (5 ng/ml) (Cyt) for 48 h, as indicated. Values for the percentage of TUNEL-stained cells are means ± SE for the number of independent experiments shown at the base of each bar. *, $P < 0.01$ *vs.* Cyt values, as determined by one-way ANOVA and multiple comparison test.

When islets were cultured for 48 h in the presence of GF109203X, a PKC inhibitor, TUNEL-stained cells increased to levels observed with cytokine treatment alone (Table 2). Moreover, the combination of cytokines with GF 109203X did not affect the induction of TUNEL staining (Table 2). Surprisingly, the combination of GF 109203X with cytokines and S1P did not prevent the marked inhibition of TUNEL staining noted with cytokines and S1P (Table 2). Thus, S1P not only inhibited cytokine-induced apoptosis/necrosis, but it also inhibited the GF 109203X-induced apoptosis/necrosis.

Cytochrome c release

The release of cytochrome c from mitochondria is one mechanism mediating the initiation of apoptosis in cells (26). INS-1 cells showed a significant elevation of cytochrome c in cytosol after exposure to IL-1β, IFN, and TNF (Fig. 7, A and B). In the presence of cytokines plus S1P, cytochrome c release was significantly lower than release observed with cytokines alone, although levels tended to be somewhat but not significantly higher than levels in basal or S1P-treated cell cytosol fractions (Fig. 7, A and B).

TABLE 1. PKC activity in INS-1 cells

Treatment	Whole cell (% of control)	Supernatant (% of control)	Particulate (% of control)
S1P	177 ± 23 (9)[a]	133 ± 22 (9)	175 ± 38 (9)[a]
PMA	397 ± 121 (8)[b]	262 ± 77 (8)[b]	370 ± 104 (7)[b]

Cells were cultured in the absence (control) or presence of S1P (400 nM) or PMA (1 μM) for 10 min, and then PKC activity was determined in whole-cell homogenates, and 100,000 × *g* supernatant or particulate subcellular fractions, as shown. Values are mean ± SE. Percent of control for the number of independent experiments shown in parentheses. PKC activity in whole-cell homogenate, supernatant, and particulate fractions was 11.6 ± 3.8, 19.0 ± 13.5, and 22.3 ± 14.6 pmol of ^{32}P incorporated into substrate peptide per minute per milligram protein, respectively.

[a] $P < 0.01$, determined by Student's *t* test (paired) *vs.* untreated control cells.

[b] $P < 0.05$, determined by Student's *t* test (paired) *vs.* untreated control cells.

TABLE 2. Effects of a PKC inhibitor, GF 109203X, on islet cell TUNEL staining

Treatments	TUNEL stain (% apoptotic cells)
Cytokines	31.6 ± 4.2
Cytokines + S1P	8.0 ± 2.9[a]
GF	37.0 ± 9.9
Cytokines + S1P + GF	7.7 ± 4.3[a]
Cytokines + GF	38.7 ± 14.5

Islets were cultured for 2 d in the absence or presence of IL-1β (1 ng/ml) and IFN (5 ng/ml) (cytokines), S1P (0.4 μM), or GF 109203X (GF) (0.5 μM). Cells were pretreated with S1P and/or GF for 4.5 h before the addition of other agents. Data are percentage TUNEL-stained cells minus basal values and are expressed as means ± SE for n = 3–5 independent experiments.

[a] $P < 0.01$ *vs.* cytokine stimulation alone, as determined by one-way ANOVA and multiple comparison test.

Caspase-3 activity

The response of INS-1 cells to S1P was investigated to determine a role for the sphingolipid in apoptotic enzyme activation. Caspase-3 activity in INS-1 cells was activated by treatment of the cells with the cytokine cocktail of IL-1β, IFN, and TNF (Fig. 8). The presence of S1P significantly reduced the cytokine-induced caspase-3 activity, although caspase-3 activity remained marginally above basal levels ($P < 0.04$) (Fig. 8). S1P alone did not significantly affect caspase-3 activity (Fig. 8). In addition, the cytokine-induced increase in INS-1 cell caspase-3 activity was completely inhibited in the presence of dihydro-S1P (Fig. 8). Dihydro-S1P did not affect the basal activity of caspase-3 (Fig. 8).

Nitric oxide synthase activity

The effect of S1P on cytokine-induced nitric oxide synthase activity in INS-1 cells was also determined. After a 24- or 48-h

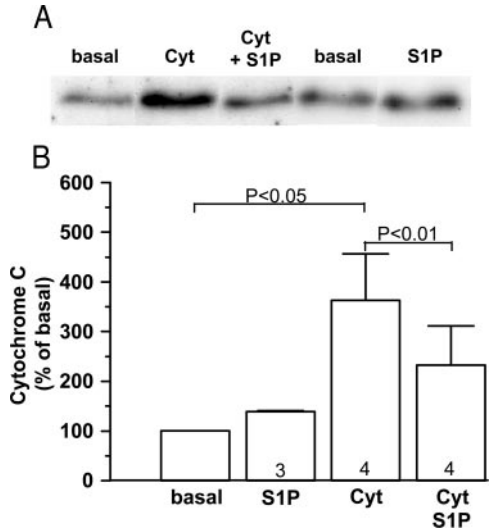

FIG. 7. S1P effects on cytochrome c release. INS-1 cells were cultured in the absence (basal) or presence of S1P (400 nM) and IL-1β (1 ng/ml), IFN (10 ng/ml), and TNF (10 ng/ml) (Cyt) for 1 d, as indicated. Cytochrome c levels in cell cytosolic subcellular fractions were determined by Western blotting. A, Representative cytochrome c Western blot. B, Values are expressed as percent of basal cytochrome c in cytosol and are means ± SE for the number of independent experiments shown at the base of each bar. *P* values determined by Student's *t* test (paired).

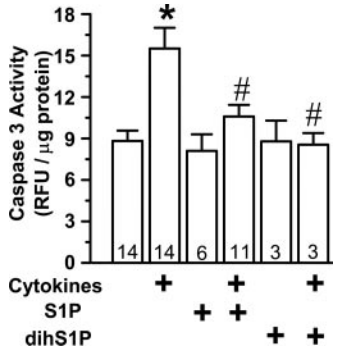

FIG. 8. S1P and dihydro-S1P (dihS1P) effects on caspase-3 activity. INS-1 cells were cultured in the absence or presence of S1P (400 nM) and a cocktail of IL-1β (1 ng/ml), IFN (10 ng/ml), and TNF (10 ng/ml) (cytokines) for 1 d, as indicated. Caspase-3 activity was determined and values are shown as means ± SE for relative fluorescence units (RFU) normalized to microgram protein. *, $P < 0.05$ *vs.* untreated control cells, #, $P < 0.05$ *vs.* cytokine-only treated samples, determined by one-way ANOVA and multiple comparison test.

regimen of IL-1β treatment in INS-1 cells, there was a time-dependent increase in inducible nitric oxide synthase production of nitric oxide (expressed as picomoles of nitrite produced) (Table 3). However, the presence of S1P only marginally reduced cytokine-induced nitric oxide synthase activity at 24 h, and no significant differences were observed after 48 h (Table 3). S1P did not significantly affect basal nitric oxide synthase activity (Table 3).

Discussion

The pancreatic islet of Langerhans β-cells are sensitive to cytokines, which induce cell death by apoptosis and necrosis (2, 4). The necrotic component of β-cell death appears to be tied to the induction of nitric oxide synthase, and nitric oxide production because mice deficient in inducible nitric oxide synthase undergo delayed apoptosis but not necrosis (5, 27). In the present study, IL-1β and IFN induced apoptosis/necrosis in cultured rat islets within 48 h, as detected by TUNEL staining. Similarly, IL-1β, TNF, and IFN induced apoptosis/necrosis in INS-1 cells. The percentage of basal TUNEL-stained cells in islets was higher than that found in INS-1 cells, perhaps due to the mechanical isolation technique required for islet cell separation and the cell sensitivity to serum withdrawal.

TABLE 3. Inducible nitric oxide synthase (iNOS) activity in INS-1 cells

Treatment groups	INS-1 cell iNOS activity (pmol nitrite/μg protein)	
	24 h	48 h
Basal	0.22 ± 0.2	0.8 ± 0.3
S1P	0.26 ± 0.2	0.5 ± 0.3
IL-1β	7.7 ± 1.3[a]	37.9 ± 6.8[b]
IL-1β + S1P	4.9 ± 0.5[a,c]	30.1 ± 6.1[b]

Cells were cultured for 24 h in serum-free RPMI 1640 containing 0.1% fatty acid free-BSA, followed by 24 or 48 h incubation in the absence or presence of IL-1β (0.1 ng/ml) or S1P (10 μM). Cells were pretreated with S1P for 4 h before the addition of other agents. iNOS activity was quantitated by determination of nitrite levels in cell culture media normalized to total cell protein levels. Values are means ± SE for four to seven independent determinations. *P* values were determined by one-way ANOVA and multiple comparison test.

[a] $P < 0.01$ *vs.* basal.

[b] $P < 0.001$ *vs.* 24-h paired values.

[c] $P < 0.05$ *vs.* 24-h IL-1β values.

S1P has been described as a second messenger in cell survival that protects cells from serum deprivation or ceramide-mediated apoptosis (19, 28). It is well known that S1P signals to cells through receptor-mediated and intracellular pathways (9). In the present study, extracellular S1P partially inhibited the appearance of apoptotic/necrotic cells in cytokine-treated islets. The effective S1P concentrations in the present study (100–400 nM) are within the physiological range found in blood (0.4–1.5 μM) (29–31). It is unlikely that a reduction in nitric oxide mediated the inhibition because S1P did not inhibit nitric oxide synthase activity in cytokine-treated INS-1 cells after 48 h. Thus, S1P appears to mediate changes in apoptosis rather than necrosis.

The effects of extracellular S1P are largely mediated by a receptor transduction mechanism. Although S1P can enter cells, the extracellular effects were confirmed by the S1P receptor agonist dihydro-S1P that lacks intracellular effects. Dihydro-S1P also inhibited the apoptotic/necrotic response to cytokines in islet cells at nanomolar concentrations. Further evidence that the S1P receptor mediates the protective effects of S1P on islet cell apoptosis/necrosis is the inhibition of the protective response by the S1P receptor antagonist BML-241 (25). Previously we reported that islets and INS-1 cells express $S1P_1$ (EDG1), $S1P_2$ (EDG5), $S1P_3$ (EDG3), and $S1P_4$ (EDG6) subtypes that are specific receptors for S1P (12). Each of the S1P receptor subtypes can be coupled to $G_{i/o}$, and $S1P_2$ and $S1P_3$ can also couple through G_q and $G_{12/13}$ (32). $S1P_1$ through $G_{i/o}$ coupling stimulates MAPK via Ras signaling, activates ERK, stimulates PLC, and inhibits adenylyl cyclase (11, 15, 33). $S1P_2$ and $S1P_3$ also couple via $G_{i/o}$ to Ras/MAPK and, unlike $S1P_1$, couple to PLC via a pertussis toxin-insensitive G_q (18, 33). The latter two receptors also couple to Rho stimulation. Another small G protein, Rac, is bimodally regulated by $S1P_1$, $S1P_2$, and $S1P_3$, with phosphatidylinositol 3-kinase mediating the $S1P_1$ and $S1P_3$ responses (32, 33). $S1P_4$ reportedly also stimulates MAPK and PLC and inhibits adenylyl cyclase (32, 34). Thus, S1P signaling through the S1P receptor subtypes present in islet cells potentially affects multiple signaling pathways regulating intracellular Ca^{2+} mobilization, cAMP levels, and MAPK activation.

We previously reported a concentration-dependent inhibition by S1P of adenylyl cyclase activation and cAMP production in islets and INS-1 cells (12). The lack of effect of pertussis toxin on the S1P-induced protection from apoptosis/necrosis, and the lack of effect of the α_2-adrenergic receptor agonist clonidine that acts through coupling to G_i, suggests that $S1P_1/G_{i/o}$ coupling is not the primary mediator of the protective response in islet cells. $S1P_2$ and $S1P_3$ receptors, mediated by G_q, are good candidates for mediating the S1P protective response in islets because dihydro-S1P binds to these receptors (10) and protects the islets from apoptosis/necrosis. In previous reports, the presence of extracellular S1P in micromolar concentrations resulted in the elevation of intracellular S1P levels that appeared to mediate protection from apoptosis in PC12 cells because dihydro-S1P did not protect these cells (11, 35). On the other hand, the protection

of endothelial cells from apoptosis by nanomolar concentrations of S1P appeared to depend on $S1P_1$ and $S1P_3$ receptor effects on ERK-2 and Rho signaling (36). Stimulation of $S1P_2$ has also been implicated in cell survival (37). Thus, S1P receptor coupling is likely to mediate islet protection in apoptosis because nanomolar concentrations of S1P are unlikely to contribute to marked elevations in islet intracellular sphingolipid, and dihydro-S1P mimics extracellular S1P.

The results also provide evidence that stimulation of the muscarinic receptor in islets by CCh can protect islets from the apoptotic/necrotic effects of cytokines. Although the extent of protection of islets from cytokine-induced apoptosis/necrosis with S1P and CCh was similar, a higher concentration of CCh (100 μM) than S1P was required. Micromolar concentrations of CCh are often required to elicit a robust PLC activation in β-cells (37) and have been reported to block apoptosis in cortical neurons (38). It is well known that stimulation of the muscarinic receptor subtypes $m_1/m_3/m_5$ in pancreatic β-cells activates G_q and PLC activation (39). In addition, the polybasic region of the short C-terminal tail of the m_3-muscarinic receptor has been identified as contributing to the ability of these receptors to mediate protection from apoptosis (40). Evidence that PLC activation might mediate the S1P protective responses was provided by the inhibitory effect of the PLC inhibitor, U73122, on S1P responses in cytokine treated islets. The results also show that S1P mimicked the effect of PMA on PKC activation in INS-1 cells. These data suggested that PLC activation, and perhaps PKC activity, mediate the S1P antiapoptotic effects. The G_q-coupled $S1P_2$ or $S1P_3$ acting through PLC stimulation, inositol 1,4,5-trisphosphate, and diacylglycerol production with resulting PKC activation likely to play roles in the S1P effects on β-cells. However, inhibition of islet PKC activity with GK 109203X induced TUNEL staining in islet cells that itself was inhibited by S1P. There is precedence for inhibition of PKC to induce apoptosis (41, 42). It remains to be determined which distal pathways mediate the S1P receptor and/or PLC-activated antiapoptotic/antinecrotic response(s) in islets. The S1P response is either more potent or efficacious than CCh in the islets, or there are additional signaling elements that contribute to the S1P response.

An early event in apoptosis is cytochrome c release from mitochrondria into the cytosol that initiates a proteolytic cascade involving caspase-3 (27) and inositol 1,4,5-trisphosphate receptors (43) that result in cell death (27). For INS-1 cells treated with cytokines, the inhibition of cytochrome c release and the reduction in the activity of caspase-3 after treatment with S1P provide evidence that S1P has specific effects on the apoptotic response *vs.* necrosis in β-cells. A low but detectable basal level of cytochrome c in cytosol may have been due to mitochondrial damage during mechanical cell disruption.

In summary, S1P receptor ligand binding initiates a signal transduction mechanism that negatively modulates the onset of cytokine-induced apoptosis in β-cells. Previously we reported that cytokines induce sphingosine kinase activity in islets and INS-1 cells (44). The increase in endogenous S1P production may be a survival response of the cells in the face of cytokine challenge. The current results suggest that extracellular S1P provides protection. It has been reported that high-density lipoprotein (HDL) particles antagonize the apoptotic response to very low-density lipoprotein in mouse islets (45). In addition, HDL and other serum lipoproteins can bind as much as 60% of the S1P in plasma (46). Moreover, the antiapoptotic actions of HDL have been correlated with the presence of S1P (47). Thus, these data showing the protective effects of extracellular S1P against cytokine-induced apoptosis suggest that S1P in blood has potential protective effects *in vivo*.

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