Activation of the Sphingomyelinase/Ceramide Signal Transduction Pathway in Insulin-Secreting β-Cells

Role in Cytokine-Induced β-Cell Death

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Activation of the sphingomyelin/ceramide pathway may mediate interleukin-1–induced β-cell death (Welsh, N: Interleuken-1β–induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF-2 in the insulin-producing cell line RINm5F. *J Biol Chem* 271: 8307–8312, 1996). In this report, we have examined this pathway in more detail. Culture of β-TC3 cells with 25 μmol/l ceramide analogs (N-acetyl- and N-hexanoyl-sphingosine) for 72 h did not significantly affect glucose- and carbachol-induced insulin secretion. Dihydroceramide (N-acetyl- or N-hexanoylsphinganine), a structurally similar analog, had no effect on agonist-induced secretion. However, ceramide analogs both time- and dose-dependently decreased cell viability, while the dihydroceramide analog had no effect. The ceramide effect on cell viability mimicked the effect of the cytokines TNF-α, IL-1β, and IFN-γ, reported stimulators of sphingomyelin hydrolysis. Cytokines, however, failed to stimulate sphingomyelin metabolism. Furthermore, using two different methods to quantitate ceramide, cytokines failed to cause an increase in β-cell ceramide content versus unstimulated or time-matched vehicle controls. Taken together, these data suggest that although ceramide analogs mimic the cytotoxic effect of cytokines, activation of the sphingomyelin/ceramide signaling pathway is not involved in cytokine-induced β-cell death. *Diabetes* 48:1372–1380, 1999

Cytokines, peptide molecules secreted by macrophages and T-cells, have been demonstrated to exert inhibitory and cytotoxic effects on pancreatic β-cells. Original observations by Mandrup-Poulsen and colleagues found supernates of stimulated nondiabetic human peripheral blood mononuclear cells cytotoxic to both rat and human β-cells in vitro (1,2). Further characterization identified interleukin (IL)-1, potentiated by tumor necrosis factor-α (TNF-α), to be the principal mediator of β-cell death (3,4). These studies suggest that if these cytokines, specifically proinflammatory cytokines, are produced by activated macrophages and T-cells infiltrating pancreatic islets during insulitis, they may serve as mediators of β-cell damage in type 1 diabetes. Several studies have confirmed the effects of cytokines on the β-cell. IL-1 inhibits insulin biosynthesis and secretion, oxidative metabolism, and results in β-cell death (5,6). The synergistic effects of TNF-α, IL-1β, and interferon-γ (IFN-γ) on β-cell toxicity have also been documented (7–9). Cytokine-induced nitric oxide (NO) production has been shown to mediate inhibitory effects on the pancreatic β-cell, predominantly insulin production and secretion (10,11). Additional studies support a role for NO in IL-1β–mediated cell death because NMMA (N^G-monomethyl-L-arginine), a competitive inhibitor of NO synthase, inhibits the cytotoxic effect (12). However, since NMMA only partially prevents the inhibitory effects of IL-1β (13), and nicotinamide prevents destruction of β-cells without inhibiting NO formation (14), NO production may be a necessary, but not sufficient, condition for cytokine-induced destruction of islet β-cells. Consequently, alternate effector mechanisms may have a role in cytokine-mediated β-cell death.

TNF-α, IL-1β, and IFN-γ activate the sphingomyelinase signal transduction pathway to mediate their effects on target cells (15,16). Several lines of evidence support a role for the sphingomyelinase pathway in mediating the effects of cytokines, and further implicate ceramide as a second messenger of cytokine action. Initial studies identified sphingomyelin turnover as an effector mechanism for the action of TNF-α and IFN-γ on cell differentiation of human promyelocytic leukemia cells (17). Additional experiments support a role for the sphingomyelinase signaling pathway in TNF-α activity (18). Similar activation of the sphingomyelinase pathway by IL-1β has been demonstrated (19,20). Further, as cell-permeable ceramides mimic the effects of IL-1β, a second messenger role for ceramide in IL-1β–mediated signaling is suggested. Recent evidence suggests that activation of the sphingomyelinase pathway by cytokines or cell stress, and increases in intracellular ceramide, activate a distinct pathway mediating programmed cell death (21–24). Accordingly,

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C2-cer, C2-ceramide, N-acetylsphingosine; C6-cer, C-6-ceramide, N-hexanoylsphingosine; IL, interleukin; IFN-γ, interferon-γ; C2-DHC, C2-dihydroceramide; DETAPAC, diethylenetriamine pentaacetic acid; MTT, C,N-diphenyl-N′4-5-dimethyl thiazol-2-yl tetrazolium bromide; NIH, National Institutes of Health; NMMA, N^G-monomethyl-L-arginine; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor-α.

cytokines have been implicated in activation of the apoptotic program in pancreatic β-cells (12,25). Data suggest that the cytotoxic effects of cytokines are, in part, independent of NO generation, and it has subsequently been shown that sphingomyelin degradation is independent of NO generation in β-cells (26). These results prompted us to further investigate the mechanism of cytokine-mediated signal transduction in the β-cell, with an emphasis on the role of the sphingomyelinase/ceramide pathway in β-cell death.

RESEARCH DESIGN AND METHODS

Materials. Recombinant mouse cytokines (TNF-α, IL-1β, and IFN-γ) were purchased from Genzyme (Cambridge, MA). D-erythro-Ceramide analogs (N-acetyl-, N-hexanoylsphingosine) and dihydroceramide analogs (N-acetyl-, N-hexanoyldihydrosphingosine) were purchased from Matreya (Pleasant Gap, PA), Calbiochem (San Diego, CA), or Biomol (Plymouth Meeting, PA). Sphingomyelinase (from *Staphylococcus aureus*) and MTT (C,N-diphenyl-N′4-5-dimethyl thiazol-2-yl tetrazolium bromide) were from Sigma (St. Louis, MO). [methyl-^{3}H]Choline chloride (81 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and [choline-methyl-^{14}C]sphingomyelin (54.5 mCi/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). The sn-1,2-diacylglycerol assay reagent system was purchased from Amersham Life Science (Arlington Heights, IL). [^{14}C]Serine (49 mCi/mmol) was purchased from ICN (Costa Mesa, CA).

Methods

β-Cell culture. β-TC3 insulinoma cells were obtained through the University of Pennsylvania Diabetes Endocrinology Research Center from Dr. D. Hanahan (University of California, San Francisco, CA). RINm5F insulinoma cells were purchased from the American Type Culture Collection (Rockville, MD). β-TC3 and RINm5F cells were cultured in complete RPMI-1640 (11 mmol/l glucose) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mmol/l L-glutamine at 37°C under conditions of 95% air/5% CO_2. The medium was changed twice weekly and on the day before an experiment. Cells were trypsinized and subcloned weekly. β-TC3 cells were used exclusively between passages 38–52, RINm5F between 23–52. For exogenous ceramide studies, indicated concentrations of ceramide analogs in ethanol vehicle (final concentration ≥0.1%) were added to complete RPMI-1640 for 24–96 h. Vehicle-treated cells served as negative controls. For cytokine studies, the indicated cytokines were added in a phosphate-buffered saline/0.1% bovine serum albumin vehicle to complete RPMI-1640. Where indicated, basal refers to untreated cells.

Static insulin secretion assays. Cultured β-TC3 cells were removed from incubation and washed three times in Krebs-HEPES buffer (25 mmol/l HEPES, pH 7.40, 115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, and 0.1% bovine serum albumin) and preincubated in the same medium for 30 min at 37°C under 95% air/5% CO_2. The preincubation buffer was aspirated, and Krebs-HEPES buffer with either 0 mmol/l glucose or 15 mmol/l glucose plus 0.5 mmol/l carbachol was added for another 30 min. At the end of the incubation period, aliquots of supernatant were centrifuged for 2 min at 100 g before insulin determination by radioimmunoassay. For normalization, the number of viable cells per well was counted using the trypan blue exclusion method. Final results were expressed as picounits of insulin per viable cell multiplied by time in minutes.

Cell viability determination. The MTT assay has been described as an indirect measure of β-cell metabolism and viability (27). The assay is based on the ability of viable cells to reduce MTT (C,N-diphenyl-N′4-5-dimethyl thiazol-2-yl tetrazolium bromide) to insoluble colored formazan crystals. After culture, cells were washed three times in Krebs-HEPES buffer 0 mmol/l glucose and preincubated for 30 min at 37°C under 95% air/5%CO_2. Cells were then incubated in Krebs-HEPES buffer with 15 mmol/l glucose and 0.5 mg/ml of MTT for 60 min. The supernatant was aspirated, and cells were lysed with 750 μl of isopropanol. Formazan crystals were allowed to dissolve at room temperature, and absorbance was read at 540 nm on a Fisher BT2000 MicroKinetics plate reader (Fisher Scientific, Pittsburgh, PA).

Measurement of sphingomyelin hydrolysis. β-TC3 cells grown in six-well dishes were radiolabeled with 5 μCi/ml of [^{3}H]choline chloride for 48–72 h in complete RPMI-1640 to label choline-containing phospholipids. Time-course labeling experiments indicated maximal label incorporation by 48 h. Cells were washed three times in complete RPMI-1640 to remove unincorporated label. Cells were subsequently incubated with or without TNF-α (1,000 U/ml), IL-1β (100 U/ml), and IFN-γ (500 U/ml) either alone or in combination. Bacterial sphingomyelinase (100 mU/ml) was used as a positive control, and phosphate buffered saline with 0.1% bovine serum albumin stabilizer was used as a vehicle control. All reagents were delivered in complete RPMI-1640 containing 1% fetal bovine serum for the indicated times. After incubation, cells were washed rapidly with 1 ml of ice-cold phosphate-buffered saline and fixed with 0.5 ml of ice-cold methanol before extracting lipids with chloroform/methanol/water (final 1:2:0.8 vol/vol). Lipid extraction was performed in silanized 13 × 100 round-bottom borosilicate tubes. Tubes were vortex-mixed (1 min), sonicated (30 min, 4°C), and vortex-mixed again (1 min). Tubes were centrifuged in a refrigerated tabletop centrifuge (15 min, 800*g*, 4°C). The lower organic phase was transferred to a clean silanized 13 × 100 round-bottom borosilicate tube using silanized Pasteur pipettes. The remaining aqueous upper phase was re-extracted twice with chloroform (1 ml), and the extracts were combined with the previous organic phase. The organic phase was washed with water (1 ml), evaporated under nitrogen in a Turbovap (Zymark, Hopkington, MA), and resuspended in 1 ml of chloroform/methanol (2:1). Samples were normalized to organic phosphate using 0.1 ml of sample volume (28). The remaining 0.9 ml was used for sphingomyelin analysis by thin-layer chromatography (TLC). In select experiments, glycerophospholipids were removed by incubation for 60 min at 37°C in 0.1 mol/l methanolic KOH, followed by re-extraction with chloroform/methanol/water as described above.

One-dimensional thin layer chromatography of sphingomyelin. Samples were evaporated under nitrogen, resuspended in 25 μl of chloroform/methanol (2:1), and spotted onto the preabsorbent zone of channeled LK6D silica gel TLC 20 cm × 20 cm plates (Whatman) that had been activated for 30 min at 110°C. Plates were developed for 120 min in chloroform/methanol/acetic acid/water (60:30:8:5, vol/vol). The radioactivity of the chromatogram was quantitated with a Berthold linear analyzer 284 (Nashua, NH) equipped with a position-sensitive proportional high-resolution counter tube (200 mm long, 1380 V) continuously flushed (0.5 l/min) with P10 gas (90% argon, 10% methane) and a 4-mm entrance window. Each TLC lane was scanned in its entirety (20 cm) for 60 min. Data analysis was performed using version 7.19 of the 1D-TLC software. Peak identity was assigned by comparison with iodine-stained standards and radiolabeled commercial sphingomyelin (choline-methyl-^{14}C). R_f values of [^{3}H]choline-labeled phospholipids were as follows: sphingomyelin, separating as two distinct peaks of R_f 0.31 and 0.32, most likely corresponding to species with different fatty acyl moieties (26); phosphatidylcholine, 0.51; and lysophosphatidylcholine, 0.22.

Enzymatic measurement of ceramide mass. Total ceramides were measured enzymatically by the diacylglycerol kinase assay described by Preiss et al. (29), with modifications for the optimization of ceramide quantitation: the assay buffer pH was increased to 7.40 to obtain optimal ceramide phosphorylation by *Escherichia coli* diacylglycerol kinase (30). Lipid extracts in silanized 13 × 100 round-bottom borosilicate tubes were dried under nitrogen and dissolved in 20 μl of detergent (7.5% [wt/vol] n-octyl-β-glucopyranoside, 5 mmol/l cardiolipin in 5 mmol/l diethylenetriaminepentaacetic acid [DETAPAC]). Diacylglycerol kinase was diluted 1:1 in enzyme diluent (0.01 mol/l imidazole/HCl, 1 mmol/l DETAPAC) in accordance with the manufacturer's instructions, and 500 μl was added to a mixture containing 2.5 ml of assay buffer (120 mmol/l HEPES [pH 7.4], 100 mmol/l LiCl, 25 mmol/l $MgCl_2$, 2 mmol/l EGTA) and 500 μl of 20 mmol/l dithiothreitol to prepare the diacylglycerol kinase reagent mix. Seventy μl of the reagent mix was added to the detergent/extract, and the reaction was incubated at ambient temperature for 10 min before addition of 10 μl of 10 mmol/l [^{32}P]ATP (0.08 Ci/mmol [0.8 μCi/μl]). The tubes were gently vortex-mixed and incubated at 25°C in a Dubnoff shaking water bath for 30 min. The reaction was terminated by the addition of 0.7 ml of 1% perchloric acid and 2 ml of ice-cold chloroform/methanol (1:2 vol/vol). Ten μg of lysophosphatidic and phosphatidic acid were added as carrier for the extraction. An additional 1 ml of chloroform and 1% perchloric acid was added, and the tubes were vortex-mixed (1 min) and centrifuged in a refrigerated tabletop centrifuge (15 min, 800 g, 4°C). Ceramide-1-phosphate (derived from ceramide) was recovered after washing the organic phase twice with 1% perchloric acid/methanol (7:1 vol/vol). Tubes were evaporated under nitrogen and resuspended in 25 μl of chloroform/methanol (2:1). Ceramide-1-phosphate was separated by one-dimensional TLC using Whatman silica gel LK-6D plates, activated for 30 min at 110°C in a solvent system of chloroform/methanol/acetic acid (65:15:5, vol/vol) for 60–70 min. TLC plates were dried and exposed under Kodak X-OMAT film (Kodak, Rochester, NY) for 18 h, and regions corresponding to ceramide-1-phosphate were scraped into 20-ml scintillation vials, eluted with 0.5 ml of methanol, suspended in 15 ml of Opti-phase Hisafe-3 scintillation fluid, and counted in a liquid scintillation spectrophotometer. To quantitate ceramide, ceramide standards were systematically run in parallel. Linear responses were obtained between 0 and 3,000 pmol. Results were normalized to organic phosphate as determined by a phosphate assay (28).

Ceramide quantitation using [^{14}C]serine labeling. β-TC3 cells (1 × 10^6 cells) were labeled in complete RPMI-1640 with [^{14}C]serine (0.2–0.5 μCi/ml) for 24 h as previously described (31). Cells were washed twice with RPMI-1640 containing 1% fetal bovine serum to remove unincorporated label, and stimulated with vehicle, a cytokine mixture, or sphingomyelinase as described above. Incubations were stopped by washing twice in phosphate-buffered saline and extracting lipids as described above. Lipid extracts were spotted on silica gel G TLC plates (Analtech, Newark, DE) and developed to 70% of plate length in chloroform/methanol/water/25% ammonium hydroxide (50:50:2:1 vol/vol). Plates were dried under continuous nitrogen and rechromatographed in chloroform/methanol/water/25% ammonium hydroxide (90:10:0.5:0.5 vol/vol). Radioactive lipids were identified by

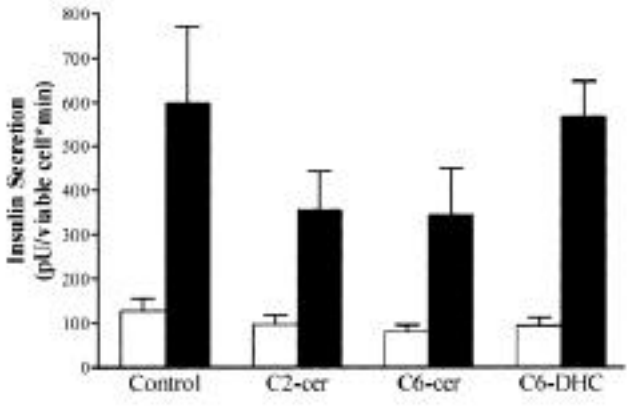

FIG. 1. Effect of ceramide analogs on β-TC3 insulin secretion. β-TC3 cells were cultured in the presence of vehicle controls (0.1% ethanol), 25 µmol/l C2-cer, C6-cer, or C6-DHC for 72 h. Static insulin secretion assays were performed in Krebs-HEPES buffer containing either 0 mmol/l glucose (□) or 15 mmol/l glucose and 0.5 mmol/l carbachol (■). Insulin secretion was determined by radioimmunoassay and normalized to viable cell number. Results are expressed as picounits of insulin per cell multiplied by time in minutes, and are representative of triplicate determinations from four independent experiments.

comparison with iodine staining of unlabeled reference standards. Ceramide was identified as two distinct spots (R_f 0.80, 0.89), indicative of the fatty acid composition of the standard (primarily stearic and nervonic acids). Under these conditions, ceramide was well separated from serine-containing lipids. Ceramide was quantitated by using a Berthold linear analyzer, and results were normalized to organic phosphate as described.

Radioimmunoassay. Insulin radioimmunoassays were performed by the University of Pennsylvania Diabetes Endocrinology Research Center.

Data analysis. For multiple comparisons between experimental groups, data were analyzed by one-way analysis of variance, followed by the Student-Newman-Keuls method. Differences were considered to be statistically significant for $P < 0.05$.

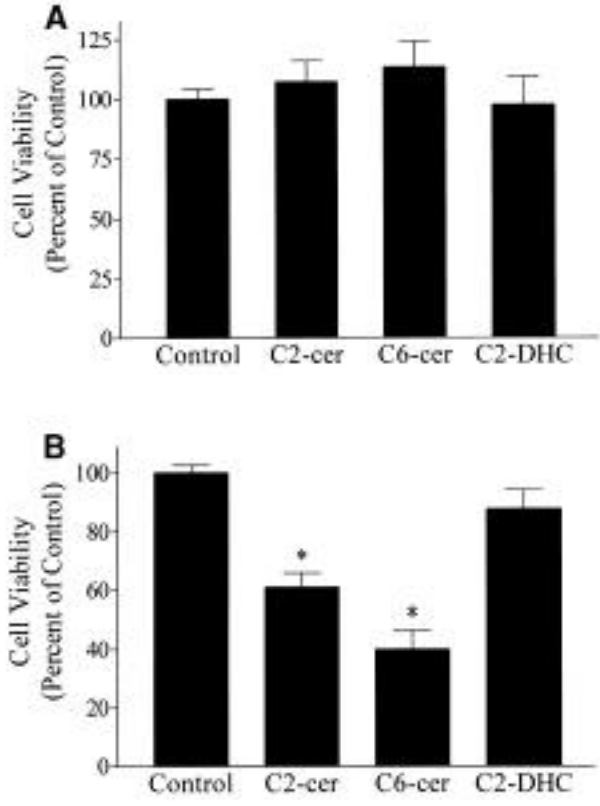

FIG. 2. Effect of ceramide analogs on β-TC3 cell viability. β-TC3 cells were cultured for 24 h (*A*) or 96 h (*B*) as in Fig. 1. After incubation, cell viability was determined by the MTT assay as described in METHODS. Results are expressed as percent of vehicle-treated controls (A_{540} 0.852 ± 0.166) and are representative of triplicate determinations from three independent experiments. *$P < 0.05$ vs. control.

RESULTS

Ceramide analogs and β-TC3 insulin secretion. Synthetic cell-permeable ceramide analogs (C2-ceramide [C2-cer] and C6-ceramide [C6-cer]) have been reported to inhibit pancreatic β-cell insulin production and mitogenesis in cultured fetal islets (32). To determine whether ceramide affected insulin secretion, β-TC3 cells were cultured with 25 µmol/l of cell-permeable ceramide analogs for 72 h. After incubation, cells were functionally assayed for their ability to secrete insulin in response to agonist stimulation. Cells were challenged with either Krebs-HEPES buffer 0 mmol/l glucose or 15 mmol/l glucose and 0.5 mmol/l carbachol for 30 min, and insulin secretion was determined by radioimmunoassay. Although results did not achieve statistical significance, data indicated an appreciable trend whereby C2-cer and C6-cer decreased agonist-induced insulin secretion 41 and 42%, respectively, versus agonist-stimulated controls, without any effect on basal insulin secretion (Fig. 1). C6-dihydroceramide (C6-DHC), which has been described as a biologically inactive ceramide analog displaying no inhibitory effects on cell growth or apoptosis (33), had no appreciable effect on agonist-induced secretion, perhaps indicating structural specificity of the ceramide versus dihydroceramide analogs.

Ceramide analogs inhibit β-TC3 cell viability. To determine whether ceramide analogs could induce cytotoxic effects similar to those reported with cytokines, β-TC3 cells were incubated in the presence of ceramide analogs (25 µmol/l), as in Fig. 1, for either 24 or 96 h. After incubation, cell viability was determined by the MTT assay. Ceramide analogs had no effect on β-TC3 cell viability after 24 h (Fig. 2*A*); this result was confirmed by the trypan blue exclusion assay (data not shown). In contrast, after 96 h, C2-cer and C6-cer decreased β-TC3 cell viability 39 and 60%, respectively ($P < 0.05$), whereas C2-DHC had no significant effect (Fig. 2*B*). Taken together, these results suggest that although ceramide analogs may decrease agonist-induced insulin secretion, ceramides may further exert a time-dependent cytotoxic effect on β-cells, as has been reported with the proinflammatory cytokines.

Dose effects of ceramide analogs on β-cell viability and cell morphology. To determine whether the cytotoxic effect observed in Fig. 2 was ceramide-dependent, β-cell viability was assessed over a micromolar range of increasing ceramide analog concentrations. Cells were cultured in the presence of cell-permeable ceramide analogs (0–100 µmol/l) for 24 h. Results indicated that C2-cer and C6-cer dose-dependently decreased cell viability, with maximal effects observed at concentrations of 100 µmol/l (Fig. 3). In contrast, 100 µmol/l C2-DHC had no significant effect. Furthermore, Fig. 4 demonstrates the effect of ceramide analogs on cell morphology. Treatment of β-TC3 cells with C2-cer and C6-cer resulted in marked morphological changes, such as cell detachment and rounding (Fig. 4*C* and *D*, respectively), whereas treatment with C2-DHC had no appreciable effect on cell morphology (Fig. 4*B*) versus vehicle-treated controls (Fig. 4*A*).

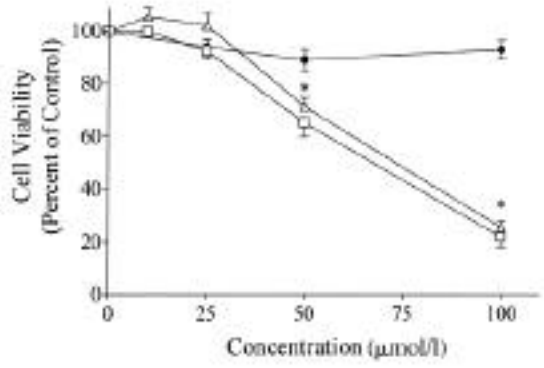

FIG. 3. Dose effects of ceramide analogs on β-TC3 cell viability. β-TC3 cells were cultured in the presence of ceramide-analogs (△, C2-cer; □, C6-cer; ●, C6-DHC) for 24 h, and cell viability was determined by the MTT assay. Results are expressed as in Fig. 2 and are representative of triplicate determinations from three independent experiments. *$P < 0.05$.

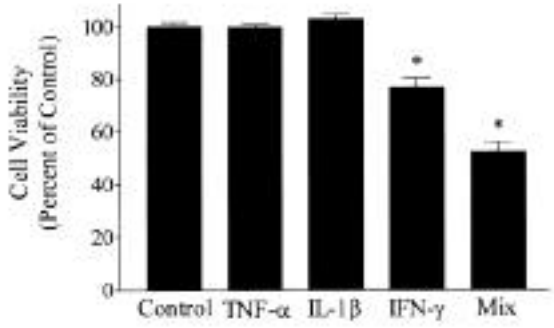

FIG. 5. Effect of cytokines on β-TC3 cell viability. β-TC3 cells were incubated for 72 h with TNF-α (1,000 U/ml), IL-1β (100 U/ml), or IFN-γ (500 U/ml) either alone or in combination (Mix). Viability was determined by the MTT assay. Results are expressed as in Fig. 2 and are representative of triplicate determinations from three independent experiments. *$P < 0.05$.

TNF-α, IL-1β, and IFN-γ inhibit β-cell viability. β-TC3 cells were assessed for their sensitivity to the cytotoxic effects of TNF-α, IL-1β, and IFN-γ. Cells were cultured with or without TNF-α (1,000 U/ml), IL-1β (100 U/ml), and IFN-γ (500 U/ml) either alone or as a cytokine cocktail for 72 h, and viability was assessed by the MTT assay as described. After 72 h of cytokine exposure, the combination of TNF-α, IL-1β, and IFN-γ decreased β-TC3 cell viability 36% ($P < 0.05$) (Fig. 5). Similar results were obtained in RINm5F cells (data not shown). The synergistic effect of cytokines on β-cell death is in agreement with published observations in rat islets (7,8).

Do cytokines increase β-TC3 sphingomyelin metabolism? The observation that ceramide analogs mimicked the cytotoxic effect of cytokines prompted us to investigate whether cytokines activated the sphingomyelinase signal transduction pathway in β-cells. Activation of sphingomyelinase has been linked to several cell-surface receptors, including the 55 kDa TNF-α receptor (18,34) and the 80 kDa IL-1 receptor (20,35), and has been reported to be an effector mechanism of IFN-γ (17). To measure cytokine-induced sphingomyelin hydrolysis, β-TC3 cells were labeled with [^{3}H]choline and subsequently incubated with TNF-α, IL-1β, and IFN-γ. Initial experiments indicated that after a 20-min exposure, cytokines, either alone or in combination, decreased β-TC3 sphingomyelin content up to 51% ($P < 0.05$) versus vehicle-treated controls (Fig. *6A*), a result in agreement with sphingomyelin hydrolysis reported in other systems (17,36). In comparison, exogenous bacterial sphingomyelinase decreased β-TC3 sphingomyelin levels by 64% ($P < 0.05$). Decreases in [^{3}H]phosphatidylcholine were also observed (Table 1). However, cytokines did not affect the flux of radioactive choline from phosphatidylcholine to sphingomyelin (data not shown). Similar, albeit less pronounced, results were found using the RINm5F cell line, a result in agreement with Welsh (37). In RINm5F cells, treatment with a combination of cytokines resulted in a 20% decrease versus control, while bacterial sphingomyelinase decreased sphingomyelin levels 19% versus control (data not shown). Initially, these data appeared to be consistent with the hypothesis that cytokines activate the sphingomyelin signal transduction pathway in β-cells. However, additional experiments examining the kinetics of sphingomyelin hydrolysis indicated that cytokine treatment may not specifically induce sphingomyelin hydrolysis. Sphingomyelin levels, in response to either vehicle or a cytokine mixture, displayed similar turnover kinetics over a 4-h time course (Fig. 6*B*). Within 2 min of experimental treatment, which consisted of washing cells three times followed by addition of reagents in unlabeled media, recovered [^{3}H]sphingomyelin content increased more than twofold over untreated (t_0) cells (vehicle- or cytokine-treated cells: 512.0 ± 40.2 or 509.3 ± 33.9 net counts/nmol phosphate, respectively, vs. untreated levels of 233.4 ± 1.6 net counts/nmol phosphate). This unexpected observation may reflect increased sphingomyelin synthesis with transfer of [^{3}H]choline from the [^{3}H]phosphatidylcholine pool to sphingosine upon medium change in β-TC3 cells. Indeed, measurement of the flux of [^{3}H]choline from the

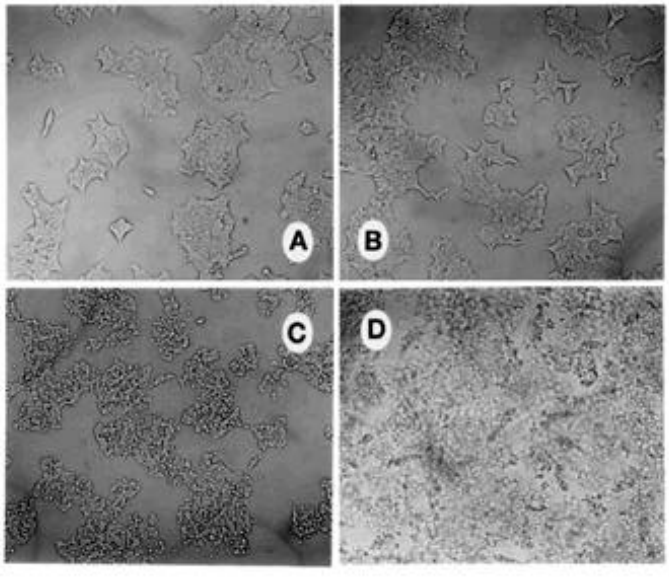

FIG. 4. Effect of ceramide analogs on β-TC3 cell morphology. Phase contrast results of cells treated as in Fig. 3. 10× magnification of cells treated for 24 h with vehicle control (*A*), 100 μmol/l of C6-DHC (*B*), 100 μmol/l C2-cer (*C*), or 100 μmol/l C6-cer (*D*). Fields are representative of three independent experiments.

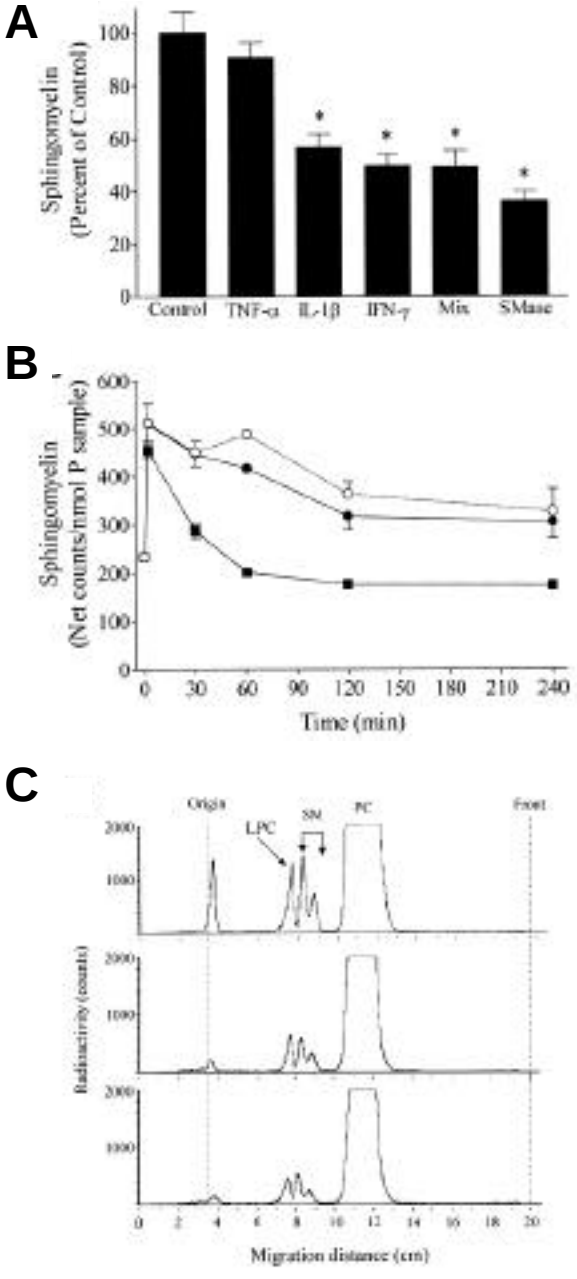

FIG. 6. Effect of cytokines on β-TC3 sphingomyelin. *A*: β-TC3 cells were radiolabeled with [^{3}H]choline. Cells were exposed to TNF-α (1,000 U/ml), IL-1β (100 U/ml), or IFN-γ (500 U/ml) either alone or in combination (Mix) for 20 min. Bacterial sphingomyelinase (SMase; 100 mU/ml) was used as a positive control. Lipids were extracted and separated by TLC. Results are expressed as percent of control labeled sphingomyelin, normalized to lipid phosphate (control values ranged from 1,219–3,499 net counts/nmol phosphate with an average experimental SD of 9.3%), and are representative of triplicate determinations from four independent experiments. *$P < 0.05$. *B*: β-TC3 cells were labeled as described and either unstimulated (t_0), or stimulated with vehicle (○), the cytokine combination (●), or sphingomyelinase (■), as above, for the indicated times. Results are expressed as [^{3}H]-incorporated sphingomyelin normalized to lipid phosphate and are duplicate determinations representative of four similar experiments. *C*: Chromatogram of [^{3}H]sphingomyelin in β-TC3 cells incubated for 20 min as in *A*. Data represent TLC of [^{3}H]sphingomyelin quantitated with the Berthold TLC-analyzer. In these experiments, sphingomyelin comprised between 4.4 ± 0.5 and 5.5 ± 0.2% of the labeled phosphatidylcholine peak. *Top panel*: vehicle-treated; *middle panel*: cytokine-treated; *bottom panel*: SMase-treated. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin.

TABLE 1
Effect of cytokines on β-TC3 phosphatidylcholine

Treatment	Phosphatidylcholine (% of control)
Control	100 ± 6.8
TNF-α	86.3 ± 5.5
IL-1β	80.8 ± 7.0
IFN-γ	54.4 ± 6.7*
Mix	42.0 ± 4.4*
SMase	39.5 ± 3.4*

β-TC3 cells were treated as in Fig. 6*A*. Data are expressed as percent of control labeled phosphatidylcholine, normalized to lipid phosphate, and are representative of triplicate determinations from three independent experiments. SMase, sphingomyelinase. *$P < 0.05$.

[^{3}H]phosphatidylcholine pool to sphingomyelin in pulse-chase experiments documented this increased flux in both control and cytokine-treated β-TC3 cells during the first few minutes of handling (data not shown). Accordingly, sphingoid base "burst," or transient increase of sphinganine and sphingosine long-chain mass with incorporation into complex sphingolipids, upon medium change has been reported in macrophages (38). A representative chromatogram of the sphingomyelin obtained in Fig. 6*A* is shown in Fig. 6*C*, demonstrating the separation of [^{3}H]choline-containing phospholipids.

To further assess the role of the sphingomyelin signal transduction pathway in cytokine-mediated β-cell dysfunction, ceramide levels in β-TC3 cells were measured by two independent methods. First, β-TC3 lipid extracts were subjected to ceramide quantitation using the modified diacylglycerol kinase assay, which measures ceramide by conversion of endogenous ceramide to ceramide-1-phosphate by reaction with diacylglycerol kinase, noting the pH dependence of diacylglycerol versus ceramide phosphorylation by *E. coli* diacylglycerol kinase (29,30). Second, ceramide was metabolically labeled with [^{14}C]serine as described by Tepper et al. (31). The rationale for these experiments was that if cytokines increased sphingomyelin metabolism, a concomitant increase in ceramide should also be observed. In contrast to results in Fig. 6*A*, vehicle or cytokine treatment had no effect on ceramide levels as measured by the modified diacylglycerol kinase assay. β-TC3 cells contained basal ceramide levels of 52.9 ± 5.3 pmol/nmol phosphate (Fig. 7*A*). Vehicle or cytokine combination treatment had no effect on ceramide levels (50.4 ± 8.0 and 54.8 ± 6.2 pmol/nmol phosphate, respectively). In these experiments, diacylglycerol mass (measured as the production of phosphatidate) did not change (data not shown). In contrast, exogenous sphingomyelinase, used as a positive control for method validation, increased ceramide mass more than fourfold to 244.2 ± 15.6 pmol/nmol phosphate. A typical autoradiogram of the ceramide assay is shown in Fig. 7*B*, as well as a representative standard curve using 0–3,000 nmol of a ceramide standard. Removal of diacylglycerol from lipid extracts by alkaline methanolysis had no effect on cytokine- or sphingomyelinase-induced ceramide generation (data not shown), indicating that ceramide conversion to ceramide-1-phosphate was not affected by endogenous diacylglycerol in

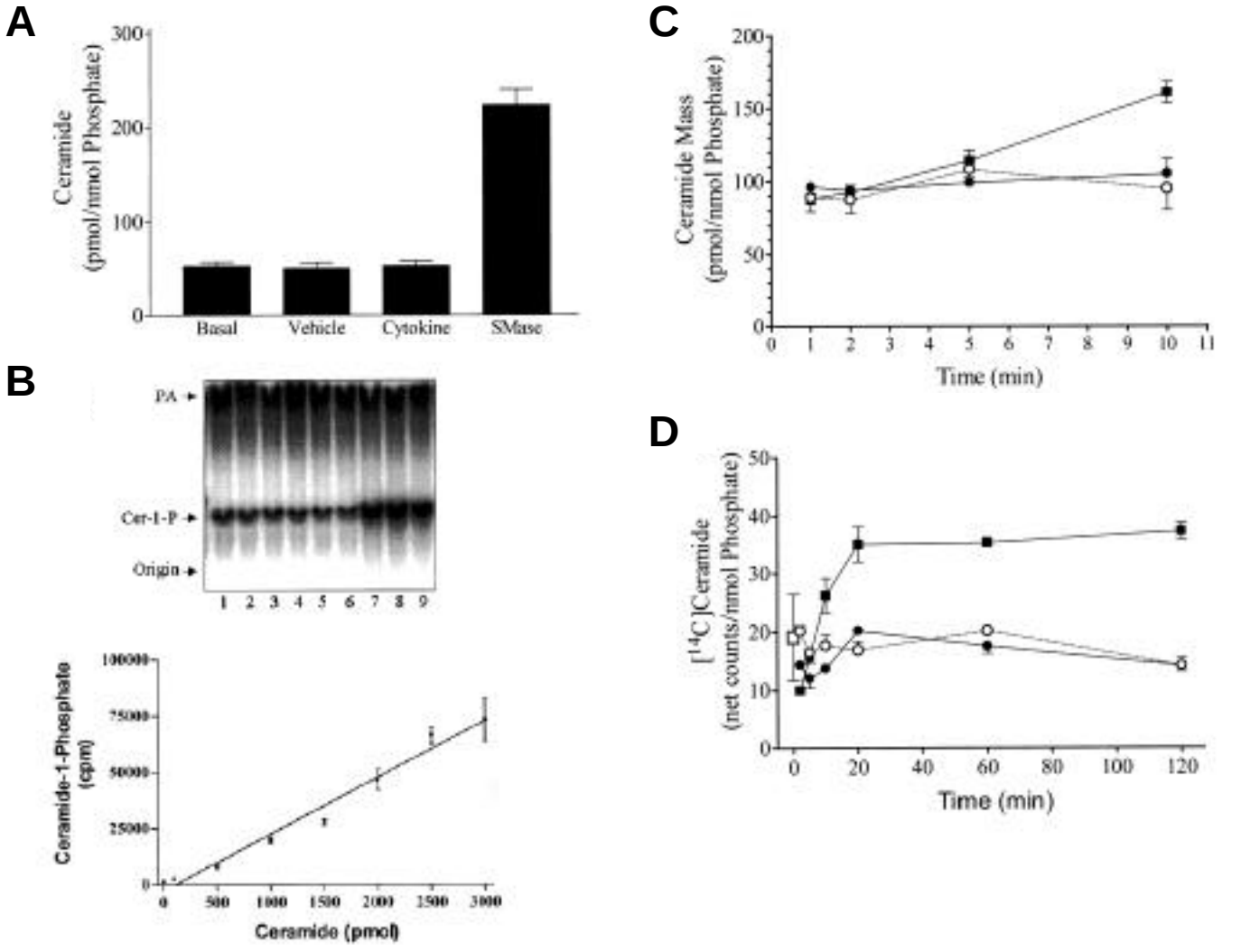

FIG. 7. Effect of cytokines on β-TC3 ceramide levels. *A*: β-TC3 cells were either untreated (basal) or treated for 20 min with vehicle, a combination of cytokines (TNF-α 1,000 U/ml, IL-1β 100 U/ml, and IFN-γ 500 U/ml), or sphingomyelinase (SMase; 100 mU/ml) for 20 min. Lipid extracts were subjected to the diacylglycerol kinase assay to quantitatively determine ceramide mass. Results were normalized to organic phosphate and represent at least triplicate determinations from three independent experiments. *B*: *Top*: representative autoradiogram from the experiment performed in *A*. The lower band represents ceramide-1-phosphate (R_f = 0.19) derived from endogenous ceramide, and the upper band represents phosphatidic acid (R_f = 0.47) derived from diacylglycerol. *Lanes 1–3*, vehicle; *lanes 4–6*, cytokine-treated; *lanes 7–9*, sphingomyelinase-treated. *Bottom*: representative standard curve using 0–3,000 nmol ceramide standard. *C*: Short-term effects of cytokines on β-TC3 ceramide accumulation. Experiment performed as in *A* with β-TC3 cells receiving treatment for the indicated time. Results are duplicate determinations from two independent experiments. *D*: Effect of cytokines on β-TC3 [^{14}C]ceramide. β-TC3 cells were labeled with [^{14}C]serine as described. Cells were stimulated with vehicle (○), the cytokine mixture (●), or sphingomyelinase (■) for the indicated times. □, basal, or t_0. Lipids were extracted, and [^{14}C]ceramide was separated by TLC. Results are expressed as net counts of [^{14}C]ceramide normalized to organic phosphate and are duplicate determinations from two independent experiments.

vitro. Similar results were obtained with the RINm5F cell line (vehicle-treated: 92.7 ± 14.3 pmol/nmol phosphate; cytokine combination: 111.0 ± 7.0 pmol/nmol phosphate; sphingomyelinase-treated: 414.0 ± 5.6 pmol/nmol phosphate). Short-term (<10 min) analysis of ceramide accumulation in response to cytokines indicated that cytokines do not acutely affect β-TC3 ceramide mass (Fig. 7*C*). In contrast, bacterial sphingomyelinase increased ceramide to 170% of time-matched controls within 10 min (161.1 ± 7.5 vs. 94.9 ± 14.6 pmol ceramide/nmol phosphate). In time-course experiments using [^{14}C]serine-labeled cells, although minor fluctuations were observed, no increase in ceramide accumulation was seen in response to cytokines over a 2-h incubation (Fig. 7*D*). In contrast, bacterial sphingomyelinase increased ceramide to 200% of basal levels (37.3 ± 1.4 vs. 19.0 ± 7.3 net counts ceramide/nmol phosphate). Taken together, these data suggest that the proinflammatory cytokines do not activate the sphingomyelin signal transduction pathway in insulin-secreting β-TC3 and RINm5F cells.

DISCUSSION

The results of this study indicate that short-chain ceramide analogs adversely affect cell viability in the insulin-producing β-TC3 cell line. Dihydroceramide, a structurally similar molecule, had no effect on agonist-induced insulin secretion or cell viability. The proinflammatory cytokines TNF-α, IL-1β,

and IFN-γ failed to increase sphingomyelin metabolism and intracellular ceramide, suggesting that ceramide may not mediate the deleterious effects of cytokines on the β-cell.

Evidence that ceramide may affect insulin secretion is supported by the observation that insulin secretion is inhibited before the onset of cell death. Furthermore, the dihydroceramide analog had no effect on agonist-induced insulin secretion or β-TC3 viability, indicating the structural specificity of ceramide. This result may possibly be explained by accumulation of ceramide analogs in the Golgi apparatus (39), which may affect insulin secretion independent of cell death. Involvement of ceramide in secretion is supported by observations by Rosenwald and Pagano (40), who described a ceramide-induced decrease in viral glycoprotein transport through the medial and Trans-Golgi complex in vesicular stomatitis virus–infected CHO cells. The effect of ceramide analogs on β-cell death was specific for ceramide, since concentrations of dihydroceramide up to 100 μmol/l had no effect on β-cell viability. These data support observations that exogenous ceramide analogs activate a distinct pathway mediating programmed cell death (21–24).

The observation that exogenous ceramide analogs mimic the effect of proinflammatory cytokines on β-cell death prompted us to investigate whether ceramide functions as a second messenger in cytokine-induced β-cell death. The sphingomyelin cycle was initially described by Okazaki et al. (36) in human lymphoma (HL-60) cells in response to vitamin D_3, whereby early and reversible hydrolysis of sphingomyelin was observed. Similar decreases in sphingomyelin upon TNF-α, IFN-γ, and IL-1β have been reported (17,18,20). In our study, initial experiments indicated that the proinflammatory cytokines, either alone or in combination, decreased [^{3}H]sphingomyelin content of insulin-secreting β-TC3 cells versus vehicle-treated controls after a 20-min exposure. These data appeared to support previous observations by Welsh (37) in the RINm5F cell line that sphingomyelin degradation occurs within 20 min of cytokine stimulation. However, similar experiments using a detailed time course produced conflicting data. Treatment of β-TC3 cells with vehicle or cytokines delivered in fresh medium resulted in increases in sphingomyelin content compared with unstimulated resting cells, and cytokines had no significant effect on sphingomyelin content compared with vehicle-treated cells. These observations suggest that the experimental procedure of washing labeled cells with RPMI-1640/1%FBS medium and adding reagents in unlabeled media increased the pool of labeled sphingomyelin, and the observed cytokine-induced decrease in sphingomyelin content versus vehicle-treated controls (seen only at 20 min) is an artifact of handling. Accordingly, Smith and Merrill (38) have reported a sphingoid base "burst," or transient increase in both sphingosine and sphinganine, in J774.1 macrophages upon changing the medium of cells in culture, which they attributed both to de novo synthesis and to turnover of complex sphingolipids. In pulse-chase experiments, we have also documented the presence of such a burst or transfer of choline from phosphatidylcholine to sphingomyelin within the first few minutes of handling the cells.

Perhaps the most significant finding of this work is that the changes in sphingomyelin do not correspond to increases in cellular ceramide accumulation. β-TC3 ceramide levels remained virtually unchanged upon cytokine exposure, using two different assays for ceramide analysis. As a positive control, bacterial sphingomyelinase increased intracellular ceramide levels more than fourfold of basal. Similarly, in RINm5F cells, cytokine treatment did not result in changes in ceramide levels. In contrast, corresponding decreases in sphingomyelin and increases in ceramide have been reported due to TNF-α and IL-1β in the HL-60 cell line (17,18,20). The results of our study are in contrast to those in a previous report by Welsh (37), which suggests that IL-1β stimulates sphingomyelin hydrolysis and ceramide generation in β-cells. To our knowledge, despite studies indicating the effect of ceramide analogs on β-cells (32,37), no quantitative decrease in sphingomyelin and corresponding increase in ceramide has been described in β-cells. Additionally, neither IL-1β nor TNF-α was found to induce hydrolysis of islet sphingomyelin species (26), further questioning the role of ceramide in cytokine-mediated β-cell death.

The discrepancy between sphingomyelin metabolism and lack of intracellular ceramide accumulation questions the involvement of the sphingomyelin pathway in β-cells, despite the observation that exogenous ceramide analogs cause β-cell death. The possibility that ceramide produced by sphingomyelin hydrolysis is released into culture medium cannot be ruled out by this study, although the observation that exogenous sphingomyelinase increased intracellular ceramide argues against this possibility. Alternately, ceramide may be further metabolized internally. Although not described in β-cells, ceramide can be converted to ceramide-1-phosphate or sphingosine via ceramide kinase and ceramidase, respectively. Although the biological role of these ceramide metabolites remains unclear, ceramide-1-phosphate has been reported to stimulate DNA synthesis and cell division (41). Sphingosine, another ceramide metabolite, is a potential inhibitor of protein kinase C (42) and has been implicated in human neutrophil apoptosis (43), while sphingosine-1-phosphate has been shown to suppress ceramide-mediated programmed cell death (44). Clearly, further elucidation of the potential involvement of ceramide metabolites in cell proliferation and death is warranted. Additionally, the fate of exogenous ceramide analogs once taken up by the β-cell remains unclear. Experiments by Ridgway and Merriam (45), using CHO cells and radiolabeled ceramide and dihydroceramide analogs, indicate that these analogs may serve as substrates for the synthesis of short-chain sphingomyelin, as well as sphingosine and sphinganine, and glucosylceramides. Further, experiments using fluorescent analogs localize to the Golgi complex as well as the plasma membrane, sites of sphingolipid biosynthesis and transport (40). Taken together, these studies indicate that results obtained using exogenous ceramide analogs should be carefully interpreted as to direct effects of ceramide action versus indirect effects on sphingolipid metabolism.

As reviewed by Hannun (46), ceramide responses range from seconds to hours, and the same inducer has generated different ceramide responses in different studies. Additionally, ceramide levels vary widely among cell systems investigated. Basal or unstimulated levels of ceramide in HL-60 cells have been reported in the range of 3.5 to 26.4 pmol/nmol of phosphate (17,47), while reported levels in U937 macrophages and human neutrophils range from 80 to 189 pmol/10^6 cells (48,49). In our experiments, ceramide levels in β-TC3 and RINm5F cells ranged between 50.4 ± 8.0 and 92.7 ± 14.3

pmol/nmol phosphate. Furthermore, reported increases in ceramide levels have been rather modest. A study by Verheij et al. (48), investigating stress-induced ceramide generation and apoptosis in U937 cells, reported X-ray–, H_2O_2-, UV-c radiation–, heat-, and TNF-α–induced ceramide increases of 50–75% within 30 min (48). In comparison, serum deprivation of MOLT-4 leukemic cells resulted in an up to 10- to 15-fold increase in ceramide after 96 h (50). Distinction between acute, intermediate, and persistent ceramide accumulation, and the specificity of such effects, requires further study.

The mechanism of cytokine action on β-cells is currently an area of active investigation. The radical nitric oxide has been described as a possible mediator of pancreatic β-cell damage in type 1 diabetes (51). The production of NO by inducible nitric oxide synthase (iNOS) damages β-cell mitochondria by inactivating iron-sulfur–containing enzymes and also inhibits DNA synthesis, which may contribute to β-cell death (52). Since cytokines induce the synthesis of numerous proteins via early- and late-response genes, elucidation of the distinct signaling pathways involved in transcriptional activation is currently under investigation.

In summary, we have shown that while exogenous ceramide analogs reproduce the effects of proinflammatory cytokines on the β-cell, cytokines do not increase ceramide accumulation commensurate with increases in sphingomyelin metabolism. Our results strongly argue against the involvement of the sphingomyelinase signal transduction pathway in cytokine-mediated β-cell dysfunction.

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