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Regulation by Sphingolipids of the Fate of FRTL-5 Cells

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Sphingolipids, including ceramide (Cer), sphingosine (Sph), and sphingosine 1-phosphate (Sph-1-P) have recently emerged as signal-transducing molecules. Functionally, a distinguishing characteristic of these lipids is their apparent participation in pro- or anti-proliferative cell regulation pathways. In this study, we examined the involvement of sphingolipids in the fate of FRTL-5 thyroid follicular cells. We first examined the effects of sphingolipids on FRTL-5 cell viability. Sph and Cer induced apoptosis, as revealed by fluorescence microscopy of TUNEL-positive fragmented nuclei and 180–300 bp DNA fragmentation on agarose gel electrophoresis while Sph-1-P was confirmed to prevent FRTL-5 cell apoptosis induced by deprivation of serum and TSH, possibly via cell surface receptors. We then analysed the metabolism of radiolabelled Sph and C_6-Cer (a synthetic cell-permeable Cer) in FRTL-5 cells by thin layer chromatography, followed by autoradiography. Sph was mainly metabolized to Cer, and then to sphingomyelin, while Sph conversion into Sph-1-P was hardly detected. These changes were not affected by stimulation of the cells with TSH. Our results indicate the involvement of sphingolipid mediators in the fate of FRTL-5 thyroid cells.

Key words: ceramide, FRTL-5, sphingosine, sphingosine 1-phosphate, thyrocyte.

Abbreviations: Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; Cer, ceramide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide; DAPI, diamido-2-phenylindole hydrochloride; PCR, polymerase chain reaction; TLC, thin layer chromatography; PLSD, Protected Least Significance Difference; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; S1Ps, Sph-1-P receptors.

Signal transduction pathways that use glycerophospholipid metabolites have been very well characterized. Recently, sphingolipids, another major class of membrane lipids, have also emerged as signal-transducing lipids (*1–4*). These include sphingosine (Sph), the fundamental backbone structure of all sphingolipids and glycosphingolipids, and its direct metabolites, such as sphingosine-1-phosphate (Sph-1-P) and ceramide (Cer) (*1–4*).

Functionally, a distinguishing characteristic of the sphingolipids is their apparent participation in pro- or anti-proliferative cell regulation pathways (*1–4*). For example, Cer is an important regulatory participant in programmed cell death (apoptosis) (*2*, *3*), while Sph-1-P induces mitogenesis and has been implicated as a second messenger in the cellular proliferation induced by platelet-derived growth factor and serum (*3*, *5*). It has been reported that the balance between the intracellular levels of ceramide and Sph-1-P and their regulatory effects on different family members of mitogen-activated protein kinase may determine cell fate (*6*). The effects of Sph on cell fate seem to be cell-type specific; Sph shows strong mitogenic effects in some cells, possibly through its conversion into Sph-1-P (*3*, *5*), while the role of Sph in apoptosis induction has been reported in other cells (*4*, *7*).

Strict regulation of cell proliferation is essential for the maintenance of tissue homeostasis, and the thyroid gland is no exception. The size of the thyroid gland changes after the addition or withdrawal of goitrogenic agents; thyroid follicular cells proliferate in response to goitrogen in goitre development, while the thyroid gland regresses (mediated in part by apoptosis) after goitrogen withdrawal (*8*). Although regulation of thyroid growth has been extensively studied and various peptides or proteins, including TSH (*9*), insulin-like growth factor (*9*) and transforming growth factor-β (*10*), have been shown to regulate thyroid cell proliferation and cell death, not much has been reported from the viewpoint of sphingolipids. In this study, we first investigated the effects of these sphingolipids on the cell fate of FRTL-5 thyroid follicular cells, and further examined the metabolism of sphingolipids in these thyroid cells.

MATERIALS AND METHODS

Materials—The following materials were obtained from the indicated suppliers: Sph-1-P and C_6-Cer (Biomol, Plymouth Meeting, PA, USA); Sph, sphingomyelin and sphingomyelinase (from *Staphylococcus aureus*)

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(Sigma, St Louis, MO, USA); pertussis toxin (Kaken Pharmaceutical Co., Tokyo, Japan); [3-^{3}H]Sph (22.0 Ci/mmol) and [3-^{3}H]C_6-Cer (22.3 Ci/mmol) (Du-Pont New England Nuclear, Boston, MA, USA). Sph and Cer were dissolved in 100% ethanol, dried under nitrogen, dispersed and sonicated in the medium employed.

Cell Culture—FRTL-5 cells were cultured in Coon's modified Ham's F12 medium containing 5% calf serum (Gibco BRL, Invitrogen Corp., San Diego, CA, USA) and a mixture of 6 hormones (6 hormones in 5% calf serum, 6H5) including 10 μg/ml of bovine insulin, 10 nM hydrocortisone, 5 μg/ml of transferrin, 10 ng/ml of somatostatin, 10 ng/ml of glycyl-L-histidyl-L-lysine acetate, and 10 mU/ml of bovine TSH. All of the hormones were obtained from Sigma. FRTL-5 cells were grown in 75 ml flasks (Falcon, Franklin Lakes, NJ, USA) and passaged by trypsinization every week. When the cells became subconfluent, they were starved in a medium containing 0.2% calf serum and 4H (without TSH and insulin) for >48 h. The cells were then treated as indicated in the same medium; TSH (10 mU/ml) was included when indicated. Not only TSH but also serum was removed to deprive growth factors. FRTL-5 cells were suspended in a medium containing 0.2% fatty acid-free bovine serum albumin (Sigma) when serum was not included in the medium.

Cell Viability Evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrasodium Bromide Assay—Mitochondrial function and hence the onset of cell death can be detected sensitively with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrasodium Bromide (MTT) dye reduction assay; MTT is a yellow substance that is cleaved by living cells to yield a dark blue formazan product. The cell viability and number are proportional to the value of absorbance measured by spectrophotometry at 570 nm. Since a significant increase in the cell number was not observed during the first 48 h of culture in the presence of TSH, as reported previously (*11*), cell viability and death could be evaluated in our MTT assay. The MTT assay was carried out according to the manufacturer's instructions (Chemicon, Chemicon International, Temecula, CA, USA). FRTL-5 cells were passaged into 96-well tissue plates (Falcon) at a cell density of 0.5×10^5/100 μl. After reactions, 10 μl MTT (5 mg/ml) was added to each sample, followed by a 4 h incubation. Then, 100 μl of isopropanol containing 0.04 N HCl was added and pipetted thoroughly. Within 1 h, the absorbance was measured on a plate reader at 570 nm with the use of a SPECTRAmax 340 (Molecular Devices, Sunnyvale, CA, USA). The experiment was performed on triplicated cultures and repeated at least three times in independent platings.

DNA Fragmentation Analysis by Fluorescent Microscopy and Agarose Gel Electrophoresis—After the reaction, FRTL-5 cells were collected by cell scrapers and washed with PBS (−). After washing once with 1 mg/ml of 4′,6′ diamido-2-phenylindole hydrochloride (DAPI) (Boehringer Mannheim, Mannheim, Germany) in methanol, the cells were stained with 1 μg/ml of DAPI in methanol for 15 min at 37°C. The cells were then washed with PBS (−) and spotted onto slides. Photographs were taken under a fluorescence microscope (Zeiss, Jena, Germany). Low molecular weight DNA was isolated as described previously (*12*). 2×10^6 cells were collected by cell scrapers and washed with PBS (−). The resulting cell pellets were resuspended in a lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% Triton X-100) and centrifuged at 13,000*g* for 20 min at 4°C to separate fragmented (soluble) DNA from intact chromatin. The supernatant was treated with 0.1 mg/ml of DNase-free RNase for 1 h at 37°C, followed by treatment with 0.2 mg/ml of proteinase K and 1% SDS for 2 h at 50°C. The DNA was then extracted with phenol and phenol/chloroform, and precipitated overnight with isopropanol at −20°C. The DNA samples were analysed with a 1.8% agarose gel.

Specific Labelling of Nuclear DNA Fragmentation by the Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling Method—After the reaction, FRTL-5 cells were collected by cell scrapers. After washing with PBS (−), the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and spotted onto silane-coated slides. Then, the cells were treated with 2 μg/ml of proteinase K for 15 min at room temperature and washed with PBS (−). After rinsing with TDT buffer (30 mM Trizma base, 140 nM sodium cacodylate, 1 mM cobalt chloride, pH 7.2), the cells were incubated with 0.3 U/μl of TDT and 0.01 mM biotin-16-dUTP (Sigma) in TDT buffer for 1 h at 37°C (*13*). The reaction was blocked with a TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. After washing with PBS (−), the cells were incubated with fluorescein-labelled streptavidin in 1% BSA/PBS containing 2 μg/ml of DAPI for 60 min at room temperature. After washing with PBS (−), the slides were sealed with 50% glycerol in PBS. Photographs were taken under a fluorescence microscope (Zeiss).

Reverse Transcriptase Polymerase Chain Reaction of S1P1-5—Total RNA was prepared from FRTL-5 cells using the total RNA isolation system, Isogen (Wako Pure Chemical Industries, Osaka, Japan), and the total RNA (1 μg) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, San Diego, CA, USA) in the presence of a random hexamer. The reverse-transcribed complementary DNA was then amplified in a Perkin-Elmer 9600R thermal cycler (The Perkin-Elmer, Norwalk, CT, USA).

The oligonucleotide primer pairs used for S1P1-5 were: S1P1, 5′-CTTCAGCCTCCTTGCTATCG-3′ (sense), 5′-GCAGGCAATGAAGACACTCA-3′ (anti-sense) (GenBank U10303); S1P2, 5′-TTCTGGTGCTAATCGCAGTG-3′ (sense), 5′- GAGCAGAGAGTTGAGGGTGG-3′ (anti-sense) (GenBank U10699); S1P3, 5′-TCAGGGAGGGCAGTATGTTC-3′ (sense), 5′-CTGACTTTCGAAGAGGATGG-3′ (anti-sense) (GenBank XM225216); S1P4, 5′-GGAGTACCTGCGCGGCATG-3′ (sense), 5′-CATGGCCTCGGACATGGACAC-3′ (anti-sense) (GenBank AW141943); S1P5, 5′-CATGCACCCATGTTCCTGCTC-3′ (sense), 5′-GATCGGCTTGCAGAAGCACAG-3′ (anti-sense) (GenBank AF233649), respectively.

The amplification was conducted over 40 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C. The polymerase chain reaction (PCR) products were resolved by electrophoresis on a 2.5% agarose gel in TBE buffer

(90 mM Tris–Borate, 2 mM EDTA, pH 8.3), and stained with ethidium bromide.

Metabolism of [^{3}H]Sph and [^{3}H]C_6-Cer in FRTL-5 Cells—FRTL-5 cells were treated with 1 μM (0.2 μCi) [^{3}H]Sph or [^{3}H]C_6-Cer. At the indicated time point, the reaction was terminated by the addition of 1.25 ml of ice-cold methanol. The lipids were extracted from the cells and medium separately by the method of Bligh and Dyer (*14*) and analysed for [^{3}H]Sph or [^{3}H]C_6-Cer metabolism as described previously (*15*). Portions of lipids obtained from the lower chloroform phase were applied to silica gel high-performance thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). The plates were then developed in butanol/acetic acid/water (3 : 1 : 1), followed by autoradiography. When indicated, silica gel areas containing radiolabelled sphingolipids were scraped off and counted by liquid scintillation counting.

Sphingomyelinase treatment of the extracted lipids was performed as described previously (*16*).

Statistics—The results shown are representative of three independent experiments, with similar results. When indicated, the results are presented as the mean ± SD of triplicate determinants from three independent experiments or as representative results from at least three independent experiments. Statistical analysis was performed using analysis of variance followed by Fisher's Protected Least Significance Difference (PLSD) for multiple samples and Student's *t*-test for paired data. $P < 0.05$ was considered significant.

RESULTS

Induction of Cell Death by Sph or Cer in FRTL-5 Cells—To examine the possible role(s) of sphingolipids in FRTL-5 cell fate, we first examined the effects of various Sph derivatives on the viability of these thyroid cells. The effects of Sph on cell fate seem to be cell-type specific; Sph shows strong mitogenic effects in some cells, while the role of Sph in apoptosis induction has been reported in others (*3–5*, *7*). Upon incubation with Sph, FRTL-5 cell death was observed not only in the absence of TSH (data not shown) but also in its presence; the cell viability of the thyroid cells challenged with 20 μM Sph for 24 h decreased to 10% of the control TSH-treated cells, as assessed by an MTT assay (Fig. 1A). Cer (*2*, *3*), formed via the sphingomyelin cycle, has been shown to be inducers of cell death in a variety of cells. Also in FRTL-5 cells, C_6-Cer (a cell-permeable synthetic Cer) induced marked cell death (Fig. 1A).

When stained with the DNA-binding dye DAPI and observed under a fluorescent microscope, fragmented nuclei were observed in Sph- or Cer-treated cells (Fig. 1B). The fragmented nuclei were positive for Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling (TUNEL) staining (data not shown), implying that there were nicks in the DNA strands of these nuclei. Furthermore, a typical pattern of DNA fragmentation of 180–300 bp was detected in Sph- or Cer-treated FRTL-5 cells using agarose gel electrophoresis (Fig. 1C). These findings indicate that the cell death induced by these sphingolipids results from apoptosis induction.

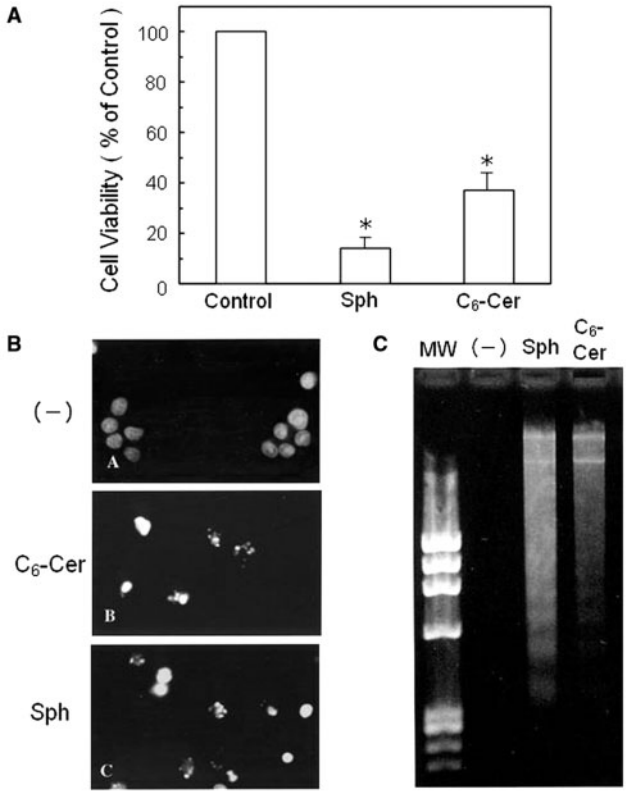

Fig. 1. **Effects of sphingolipids on the viability of FRTL-5 cells.** (A) FRTL-5 cells were treated without (control) or with 20 μM C_6-Cer or Sph for 24 h in the presence of TSH, and processed for an MTT assay (see MATERIALS AND METHODS section). Cell viability was determined as the percentage of absorbance for control cells without treatment. Asterisk denotes statistical significance (ANOVA followed by Fisher's PLSD, $P < 0.0001$) compared with control. (B) Nuclear morphological features of cell death induced by sphingolipids. FRTL-5 cells were treated without (−) or with 20 μM C_6-Cer, or Sph for 24 h in the presence of TSH, and stained with 1 μg/ml of DAPI in methanol. (C) Agarose gel electrophoresis of DNA. FRTL-5 cells were treated without (−) or with 20 μM Sph or C_6-Cer for 24 h in the presence of TSH. MW represents a 78 bp DNA molecular weight marker.

Sph-1-P Acts as a Survival Factor for FRTL-5 Cells in a Cell Surface Receptor-mediated Manner—Apoptosis was induced by deprivation of growth factors, and TSH was found to prevent apoptosis and act as a survival factor in our previous study (*11*). Of the sphingolipids, Sph-1-P, which is a metabolite of Sph by Sph kinase, has been implicated as a survival factor or a growth stimulator in various types of cells (*3–6*). Here, we confirmed that Sph-1-P prevented FRTL-5 cell apoptosis induced by the deprivation of serum and TSH, as evaluated by DAPI staining (Fig. 2A) and DNA agarose electrophoresis (Fig. 2B).

Sph-1-P is a unique bioactive lipid which acts both extracellularly as a ligand for cell surface receptor(s) (*17*, *18*) and intracellularly as a second messenger (*5*, *6*, *18*). In FRTL-5 cells, the effect of Sph-1-P on prevention of apoptosis (induced by deprivation of serum and TSH) was at least partly blocked by pertussis toxin, implying G_i-coupled cell surface receptor(s) involvement (Fig. 3).

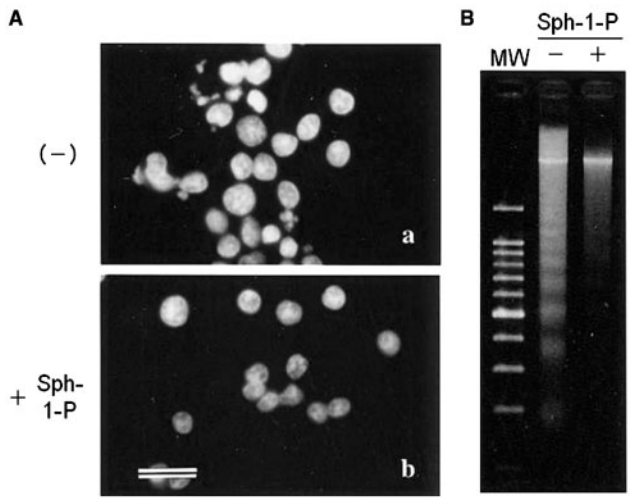

Fig. 2. **Suppression by Sph-1-P of FRTL-5 cell apoptosis induced by deprivation of serum and TSH.** (A) Nuclear morphological features of DAPI-stained FRTL-5 cells. FRTL-5 cells (cultured in 4H) were challenged without (−) or with Sph-1-P for 48 h, stained with 1 μg/ml of DAPI, and observed under a fluorescence microscope. (B) Agarose gel electrophoresis of DNA from FRTL-5 cells (cultured in 4H) treated without (−) or with Sph-1-P. MW represents a 100 bp DNA molecular weight marker.

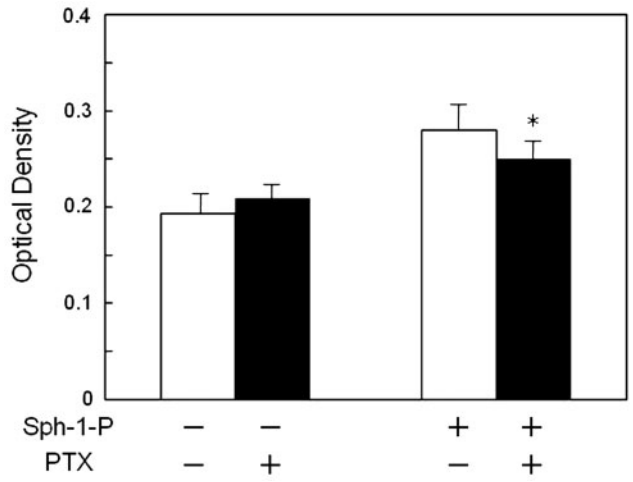

Fig. 3. **Action of Sph-1-P as a FRTL-5 cell survival factor and its inhibition by pertussis toxin.** FRTL-5 cells were pre-treated without (open bars) or with (solid bars) 50 ng/ml of pertussis toxin (PTX) for 24 h, and further cultured with or without Sph-1-P for 48 h in the absence of TSH. The cells were then processed for an MTT assay (see MATERIALS AND METHODS section). Asterisk denotes statistically significant (Student's *t*-test, $P < 0.05$) compared with the control cells (without PTX pre-treatment).

In fact, of Sph-1-P receptors (S1Ps), S1P1-4, but not S1P5, was expressed in the FRTL-5 cells, at least under our conditions employing Reverse Transcriptase PCR (RT-PCR) (Fig. 4).

Metabolism of Radio-labelled Sphingolipids in FRTL-5 Cells—Sphingolipids, derived from the sphingomyelin signalling pathway, have been demonstrated to be

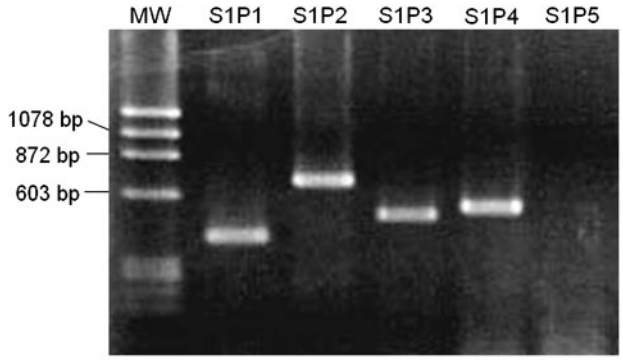

Fig. 4. **Expression of receptors for S1P in the FRTL-5 cells.** The amplified products for the FRTL-5 mRNAs for S1P1-5 were resolved in a 2.5% agarose gel. Lane MW contained the size standards (bp) as indicated. The specific amplified products for S1P1, -2, -3, -4 and -5 are 409, 695, 507, 542 and 670 bp, respectively.

involved in the regulation of cell growth and death, and FRTL-5 cells seem no exception, as described above. Since metabolism of these sphingolipids has not been reported in thyroid cells, we examined this by the analysis of lipids extracted from FRTL-5 cells incubated with radio-labelled sphingolipids. We first examined the metabolic fate of Sph in FRTL-5 cells by exogenous addition of [^{3}H]Sph into the cell culture. The added [^{3}H]Sph was rapidly incorporated into the cells, and rapidly converted to [^{3}H]Cer (by *N*-acylation) (*19*), and further to [^{3}H]sphingomyelin (through the action of sphingomyelin synthase) (*20*) (Fig. 5A). In contrast, [^{3}H]Sph conversion into [^{3}H]Sph-1-P was weak and transient; the weak [^{3}H]Sph-1-P band could no longer be observed 1 h after the label addition (Fig 5A), possibly due to degradation by Sph-1-P lyase (*21*). As described above, [^{3}H]Cer and [^{3}H]sphingomyelin were markedly formed, but only a weak and transient [^{3}H]Sph-1-P synthesis was observed in FRTL-5 cells incubated with [^{3}H]Sph. These [^{3}H]Sph conversions were not affected by TSH (Fig. 5B and C).

We finally examined the metabolism of radio-labelled C_6-Cer. One clear radioactive band was detected in the extract of FRTL-5 cells incorporating [^{3}H]C_6-Cer (Fig. 6). This band was found to be located just below sphingomyelin on TLC, and was specifically eliminated by treatment with sphingomyelinase (data not shown). These results indicate that [^{3}H]C_6-sphingomyelin is formed from [^{3}H]C_6-Cer in FRTL-5 cells. This is consistent with the fact that [^{3}H]sphingomyelin was formed later than [^{3}H]Cer when FRTL-5 cells were incubated with [^{3}H]Sph (Fig. 5). TSH again failed to affect this [^{3}H]C_6-sphingomyelin production from [^{3}H]C_6-Cer (Fig. 6).

DISCUSSION

As has been reported for a variety of cells (*1–6*), Cer induced apoptosis, while Sph-1-P prevented apoptosis in FRTL-5 cells. The roles of Sph in cell fate are cell-type specific or phenomenon specific and even contradictory effects have been reported (*3–5*, *7*). It was reported that Sph acts as a mitogen or survival factor in some

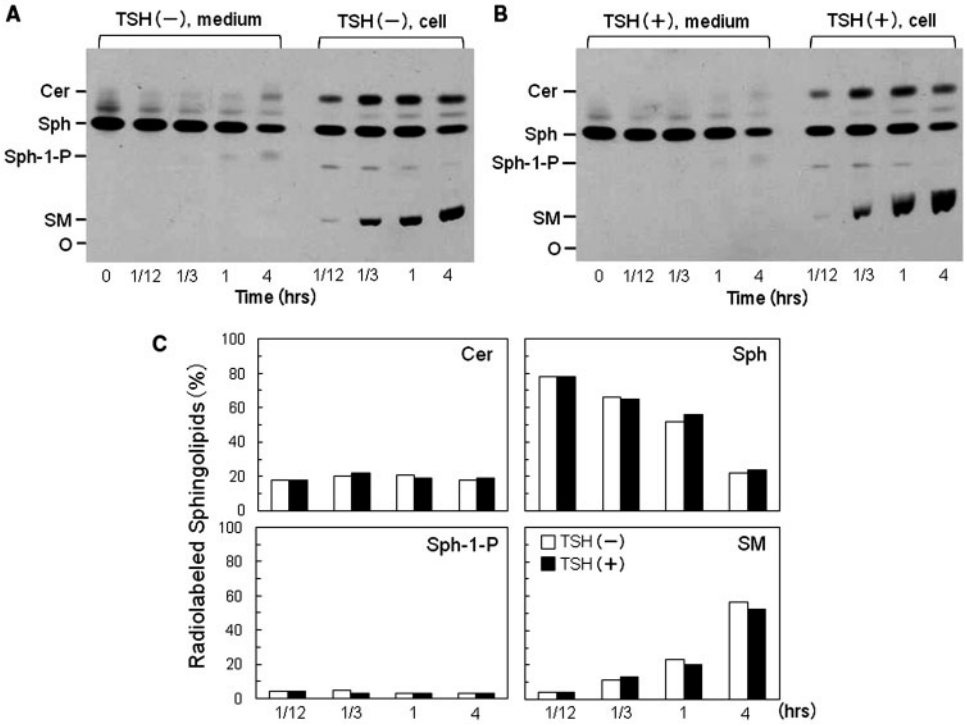

Fig. 5. **Metabolism of [^{3}H]Sph in FRTL-5 cells.** FRTL-5 cells were incubated with [^{3}H]Sph in the absence (A) or presence (B) of TSH for the indicated times. Lipids were extracted from cells and media separately and analysed for [^{3}H]sphingolipids by TLC autoradiography. Locations of standard lipids are indicated on the left. SM, sphingomyelin; O, origin. (C) The [^{3}H]sphingolipids formed were expressed as percentages of [^{3}H]Sph incorporated into cells.

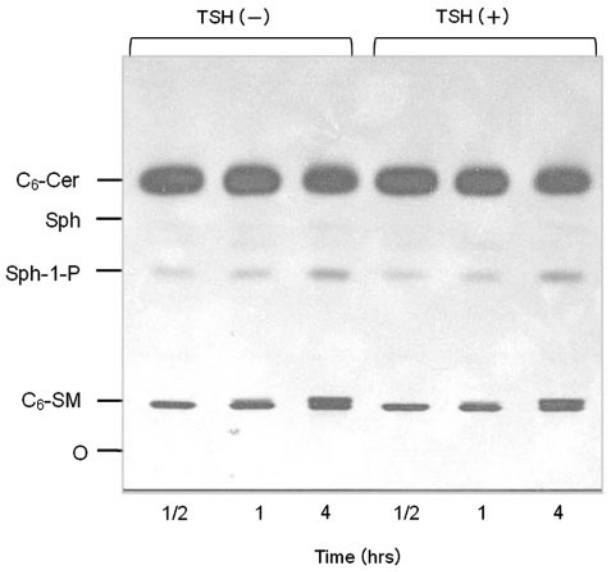

Fig. 6. **Metabolism of [^{3}H]C_6-Cer in FRTL-5 cells.** FRTL-5 cells were incubated with [^{3}H]C_6-Cer in the presence or absence of TSH for the indicated times. Lipids were extracted from the cells, and analysed for [^{3}H]C_6-Cer metabolism. Locations of standard lipids are indicated on the left.

cells, possibly via its conversion to Sph-1-P by Sph kinase (*3*, *5*). In other cells, however, Sph has been shown to be an apoptosis inducer (*4*, *7*). In FRTL-5 cells, Sph induced apoptosis. As clearly shown in Fig. 5, Sph conversion into Sph-1-P, reflecting Sph kinase activity, is weak in these thyroid cells. This may be consistent with Sph induction of apoptosis, not survival or proliferation, in these cells. Although Sph was actively converted into Cer, which is an established apoptosis inducer (*2*, *3*), it is unlikely that Sph exerts its apoptotic action through its conversion into Cer because the effect of Sph is stronger than that of Cer in these thyroid cells. In fact, the mechanism(s) by which Sph induces apoptosis is in general considered to be different from that of Cer (*4*).

Sph-1-P has been implicated not only as an extracellular mediator but also as an intracellular second messenger (*3*, *5*, *6*, *17*, *19*). In the case of FRTL-5 cell survival, Sph-1-P seems to act as a ligand for cell surface receptor(s). This is because of the following: (i) the Sph-1-P-induced anti-apoptosis effect was blocked in part by pertussis toxin, while S1Ps were confirmed to be expressed; (ii) TSH, a potent growth factor for FRTL-5 cells, failed to affect Sph-1-P formation (from Sph). Furthermore, Sph-1-P reportedly stimulates hydrogen peroxide generation (through activation of the phospholipase C-Ca2+ system), Na+/H+ exchange and DNA synthesis; at least some of the responses are sensitive to pertussis toxin (*22–24*). Presumably, FRTL-5 cells fail to use Sph-1-P, produced endogenously or intracellularly, as a pro-proliferative metabolite, which is consistent with the fact that the metabolic formation of Sph-1-P is not active in these cells. The bioactive lipid Sph-1-P acts as an important agonist to thyroid cells through cell surface receptors. On the other hand, Sph and Cer may regulate PI3 kinase/Akt in FRTL-5

cells and hence induce apoptosis since it has been shown that the PI3 kinase/Akt pathway is involved in the cell fate of these thyroid cells (*25*, *26*).

As revealed in the present investigation, Sph derivatives had different effects on the fate of FRTL-5 cells, despite the similarity in their structures. Although further studies are needed to assess the (patho)physiological implications of our findings, it is worthy of note that Sph-1-P is a normal constituent of plasma and serum, and could be released from platelets into extracellular spaces in response to various types of physiological or pathological stimulation (*4*, *16*, *27*). It is possible that variations in the plasma level of Sph-1-P may be related to clinical disorders of the thyroid gland.

CONFLICT OF INTEREST

None declared.

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