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Sphingolipids in Macroautophagy

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Summary

Sphingolipids are constituents of biological membranes. Ceramide and sphingosine 1-phosphate (S1P) also act as second messengers and are part of a rheostat system, in which ceramide promotes cell death and growth arrest, and S1P induces proliferation and maintains cell survival. As macroautophagy is a lysosomal catabolic mechanism involved in determining the duration of the lifetime of cells, we raised the question of its regulation by sphingolipid messengers. Using chemical and genetic methods, we have shown by GFP-LC3 staining and analysis of the degradation of long-lived proteins that both ceramide and S1P stimulate autophagy.

Key Words: Autophagy; ceramide; proteolysis; sphingosine 1-phosphate; sphingosine kinase; sphingolipids.

1. Introduction

The metabolism of sphingolipids is a highly dynamic process generating second messengers that include ceramide and sphingosine 1-phosphate (S1P) ***(1)***. The formation of ceramide (an N-acylated sphingoid base) is followed by a deacylation to generate sphingosine, which is phosphorylated by sphingosine kinases (SK) to produce S1P. It is now generally admitted that ceramide and S1P have contrasting roles in the response to cell stress ***(2)***. Whereas ceramide is generally associated with cell growth arrest and cell death induction, S1P promotes cell proliferation and maintains cell survival.

Macroautophagy (hereafter referred to as "autophagy") is a lysosomal, catabolic pathway regulating the turnover of macromolecules and organelles ***(3)***.

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Its impact on cell lifetime remains unclear, since autophagy appears to be a cytoprotective mechanism (when nutrient supply is limited), but can also lead to type 2 cell death (also known as autophagic cell death) as distinct from apoptosis or type 1 cell death ***(4)***.

The similar functions of the sphingolipid rheostat and of autophagy in determining cell fate led us to wonder whether sphingolipids could regulate autophagy. To find out, we investigated autophagic capacities after manipulating the levels of ceramide and S1P in MCF-7 cells and HT-29 cancer cells ***(5,6)***. The endogenous ceramide level was increased by feeding cells with the short cell-permeant C2-ceramide, whereas its biosynthesis was prevented by treating cells with the ceramide synthase inhibitor fumonisin B1 (FB1). Overexpression of sphingosine kinase 1 (SK1) was used to increase the level of S1P, and treatment with dimethylsphingosine (DMS) was used to inhibit its biosynthesis. Autophagic parameters were evaluated by measuring long-lived protein degradation as described in the following sections to estimate the autophagic flux into the lysosome and determine the number of autophagosmes formed using GFP-LC3 ***(7,8)***.

The findings show that increases in both endogenous ceramide and S1P levels can indeed trigger autophagy.

2. Materials

2.1. Cell Culture and Treatment

1. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Fisher Biosciences, ILLkirch, France) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Fisher Biosciences) and 1% penicillin-streptomycin (Invitrogen, Fisher Biosciences).
2. C_2-Ceramide (C_2-Cer, Calbiochem 110145), C_2-dihydroceramide (C_2-DHCer, Calbiochem 219537), and dimethylsphingosine (DMS, Calbiochem 310500) dissolved in ethanol at 117 m*M*, 117 m*M,* and 75 m*M*, respectively.
3. 3-Methyladenine (3-MA, Sigma M9281) and fumonisin B1 (Sigma F1147) dissolved in water before use at 10 m*M* and 5 m*M*, respectively.

2.2. Degradation of Long-Lived Proteins

1. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, pH 7.3.
2. Hanks' balanced salt solution without sodium bicarbonate (HBSS) or Earle's balanced salt solution (EBSS) from Invitrogen, Fisher Biosciences.
3. L-[U-^{14}C] Valine (266 mCi/mmol, Amersham Biosciences).
4. Trichloroacetic acid (TCA) (v/w 100%).
5. 0.2 *N* NaOH.

2.3. Lipid Phosphate Evaluation

1. Methanol and chloroform.
2. Sodium phosphate, monobasic, anhydrous (NaH_2PO_4), dissolved in water. Store at room temperature.
3. Washing buffer: 10 *N* H_2SO_4-70%$HClO_4$-H_2O, 9:1:40, (v/v/v) respectively. Store at room temperature.
4. Ammonium molybdate (Sigma), 0.9% (w/v) dissolved in water.
5. L-Ascorbic acid, 9%, (w/v) dissolved in water, prepare freshly just before use.

2.4. Preparation of Octyl-β-D-Glucopyranoside: Dioleoylphosphatidylglycerol (βOG-DOPG) Mixed Micelles

1. Octyl-β-D-glucopyranoside (βOG) (Calbiochem 494459).
2. Pyrex sintered glass funnel from E. Pasquali Srl (Italy).
3. Diethyl ether (Fluka 32203).
4. L-α-Dioleoylphosphatidylglycerol (DOPG sodium salt) (Avanti Polar Lipids 840475).

2.5. Diacylglycerol Kinase (DGK) In Vitro Assay

1. Sn-1,2-Diacylglycerol kinase, recombinant, *Escherichia coli* (≥2 units/mg protein, MW 13,700, Calbiochem 266724).
2. 2x buffer: 0.1 *M* imidazole (Sigma I0125), pH 6.6, 0.1 *M* LiCl (Sigma L8895), 25 m*M* $MgCl_2$ anhydrous (Sigma M8266), 2 m*M* EGTA (Sigma E4378), pH 6.6 dissolved in water. Store at 4°C up to 6 mo.
3. Dithiothreitol (DTT) (Sigma D5545) dissolved in water at 1 *M*; aliquots stored at –80°C.
4. Assay mixture: 50 µL of 2x buffer, DTT 3 m*M* (0.2 µL of a 1 *M* solution) and 5 µg of diacylglycerol kinase, Recombinant enzyme for each sample added just before use.
5. Dilution buffer: 10 m*M* imidazole, pH 6.6, 1 m*M* diethylenetriaminepentaacetic acid (DTPA, Sigma), pH 7.0. Store at 4°C for up to 6 mo.
6. ATP from (Amersham Pharmacia Biotech) dissolved in water at 20 m*M* and aliquots stored at –80°C.
7. Adenosine 5′-[γ-^{32}P]triphosphate, triethylammonium salt (3 Ci/µmol specific activity) from Amersham Biosciences.
8. ATP mixture: ATP, 10 m*M* and [γ−^{32}P]ATP (about 0.13 µCi/µL) for each sample, added just before use.
9. Ceramide from porcine brain used for internal standard purchased from Avanti Polar Lipids Inc. (860052).
10. Diacylglycerol used for internal standard (Avanti Polar Lipids Inc. (800811)).
11. Recrystallized octyl-β-D-glucopyranoside (βOG):

    a. Add 5 g of βOG to 20 mL of acetone.
    b. Heat mixture at 40°C to completely dissolve the βOG.

c. Filter the solution through a sintered Pyrex glass funnel.
d. Slowly add 100 mL diethyl ether to the filtered solution and reheat if necessary (if precipitation appears in the solution).
e. Place in –20°C freezer overnight (or longer) to form crystals.
f. Decant the solution containing recrystallized βOG through a sintered glass funnel.
g. Wash the crystals with 500 mL ice-cold diethyl ether.
h. Completely dry the crystals and scrape out of the funnel.
i. Weigh the crystals and store in powder form at –20°C for up to 12 mo.

2.6. Sphingosine Kinase Activity and Sphingosine 1-Phosphate Production

1. D-*erythro*-Sphingosine (Biomol EI-155) dissolved in ethanol at 50 m*M*, in a screw-cap glass tube, and stored at –20°C.
2. [γ-^{32}P]Adenosine 5´-triphosphate ([γ-^{32}P]ATP, 6000 Ci/mmol, PerkinElmer Life Sciences).
3. D-*erythro*-[3-^{3}H]Sphingosine (23 Ci/mmol, PerkinElmer Life Sciences) dissolved in ethanol.
4. Sphingosine kinase buffer : 20 m*M* Tris-HCl pH 7.4, 20% glycerol, 1 m*M* β-mercaptoethanol, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonylfluoride, 15 m*M* NaF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 40 m*M* β-glycerophosphate, 0.5 m*M* deoxypyridoxine, and 1 m*M* sodium orthovanadate.

2.7. Separation of Reaction Products (Ceramide 1-Phosphate) by Thin-Layer Chromatography

1. Thin-layer chromatography (TLC) plates from Whatman (4865-821, LK6D Silica Gel 60A, Size 20 × 20, thickness 250 μm).
2. Acetic acid (Fluka 27221) and acetone (Fluka 00570).
3. X-Omat AR from Kodak (1651454, size 20.2 × 25.4).
4. Transfer a mixture of chloroform–acetone–methanol–acetic acid–water (10:4:3:2:1, by vol.) into a well-sealed TLC chamber (use silicone and apply pressure in order to obtain good adherence of the lid). Saturate the chamber with vapors by using a sheet of filter paper as a wick (a circular strip, approximately the same height as the tank). For a 26 × 20 × 7 cm chamber, use 150 mL of solvent and allow to saturate for 5–12 h.

2.8. Separation of Reaction Products (Sphingosine 1-Phosphate) by TLC

1. Solvent A: 1-butanol/methanol/acetic acid/distilled water (80:20:10:20, by vol.)
2. Whatman LK6D TLC plates (20 cm × 20 cm).

3. Chromatography tank, containing about 100 mL of solvent A. The tank should be prepared (and sealed) at least 2 h before use.
4. Autoradiography films (Kodak BioMax MR).

3. Methods

3.1. Assay of Long-Lived Protein Degradation

The protocol described below was originally validated in human colon cancer HT-29 cells, and then in various different cancer cells, in particular in human breast cancer MCF7 cells ***(5,6)*** **(Fig. 1)**, and can be optimized depending on the cell system used. Autophagy has been also assayed after transfection of the autophagy marker **(Fig. 1)**. For a description of this method *see* **refs. *7*** and ***8***.

1. Cells are seeded in 6-well plates at 10^6 cells/plate and used near confluence.
2. Intracellular proteins are labeled for 18 h at 37°C with 0.2 µCi/mL of L-[U-^{14}C]valine in complete medium.
3. Any unincorporated radioactivity is removed by rinsing three times with PBS.
4. Cells are then incubated with fresh complete medium and 10 m*M* valine for one hour (*see* **Note 1**).
5. Short-lived proteins are degraded after incubating for one hour, when the medium is replaced; the cells are then incubated in HBSS (or EBSS) plus 0.1% of bovine serum albumin (*see* **Note 2**) and 10 m*M* valine to stimulate autophagy or with the appropriate fresh complete medium, and incubated for a further 4 h. Throughout the chase period, 3-MA can be added to inhibit de novo formation of autophagic vacuoles (***9***; *see also* **Note 3**). Under the experimental conditions shown in **Fig. 2**, the chase medium was complete medium supplemented with 10 m*M* valine **(Fig. 1B)**.
6. The medium is then precipitated overnight with TCA (100%) added to produce a final concentration of 10%.
7. After centrifuging the culture medium for 10 min at 470*g* at 4°C, the acid-soluble radioactivity is measured by liquid scintillation counting.
8. The cells are washed twice with cold 10% TCA, dissolved at 37°C in 0.2 *N* NaOH. Radioactivity is then measured by liquid scintillation counting. The rate of long-lived protein degradation is calculated from the ratio of the acid-soluble radioactivity in the medium to the acid-precipitable cell fraction.

3.2. Quantification of Ceramide

The nonpolar properties of ceramide mean that it has to be extracted from cells in organic solvents. This is done using a modification of the Bligh and Dyer method ***(10)***, which involves lysis of the cells with an organic solvent followed by dilution with chloroform and water to obtain phase separation. To obtain a single-phase mixture, it is important to maintain the following

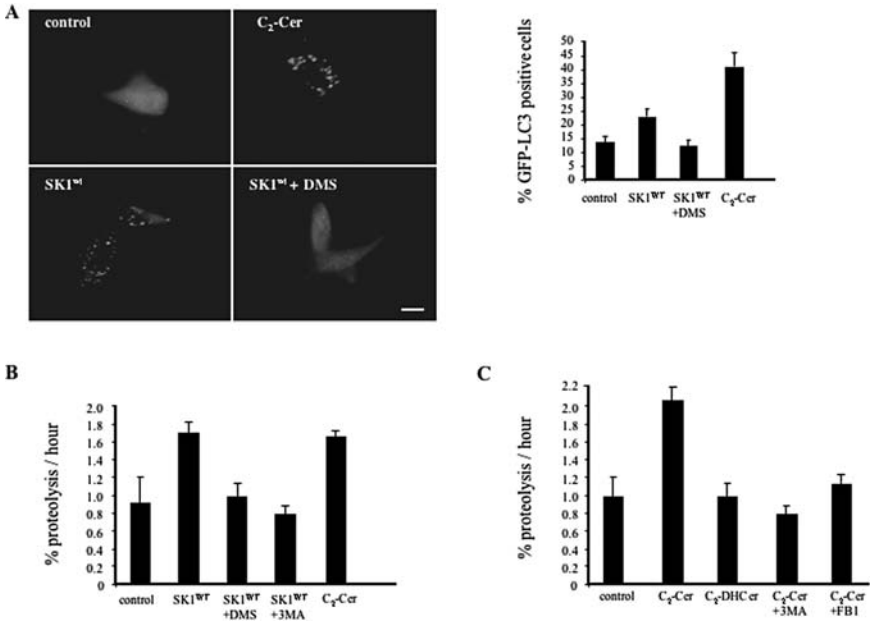

Fig. 1. Effect of sphingolipids on autophagy. **(A)** GFP-LC3 staining and quantification of cells with GFP-LC3 dots in MCF-7 cells transfected with an empty vector (control), transfected with a cDNA encoding the wild-type sphingosine kinase 1 ($SK1^{wt}$). Control cells were treated with C_2-Cer (75 μ*M*) for 2 h. When required, the cells were treated with DMS (1.5 μ*M*). The bar represents 10 μm. **(B, left)** Degradation of [^{14}C]valine-labeled, long-lived proteins in MCF-7 cells transfected with an empty vector (control), transfected with a cDNA encoding the wild-type sphingosine kinase 1 ($SK1^{wt}$). Control cells were treated with C_2-Cer (75 μ*M*) for 2 h. When required, cells were treated with DMS (1.5 μ*M*). **(B, right)** Degradation of [^{14}C]valine-labeled, long-lived proteins in HT-29 cells treated with C_2-Cer (75 μ*M*) or C_2-DHCer (75 μ*M*) for 2 h in the presence or the absence of FB1 (100 μ*M*) or 3MA (10 m*M*). (Reproduced from **refs. *5*** and ***6*** with permission from ASBMB.)

proportions: methanol, chloroform, water—2:1:0.6, (by vol.). A double-phase system is then obtained by adding chloroform and water in order to achieve a final methanol, chloroform, water ratio of 2:2:1.6 (by vol.). The lower phase, containing less polar lipids, is separated from the upper phase, containing more polar lipids plus nonlipidic molecules. The critical parameters of the lipid extraction are the ratios of chloroform, methanol, and buffer/water. Although the absolute volumes may be changed to reflect the amount of mass (tissues) being extracted, it is important that the ratios be maintained at 2:1:0.6 (by vol.) and 2:2:1.6 (by vol.) before and after dilution, respectively ***(10)***.

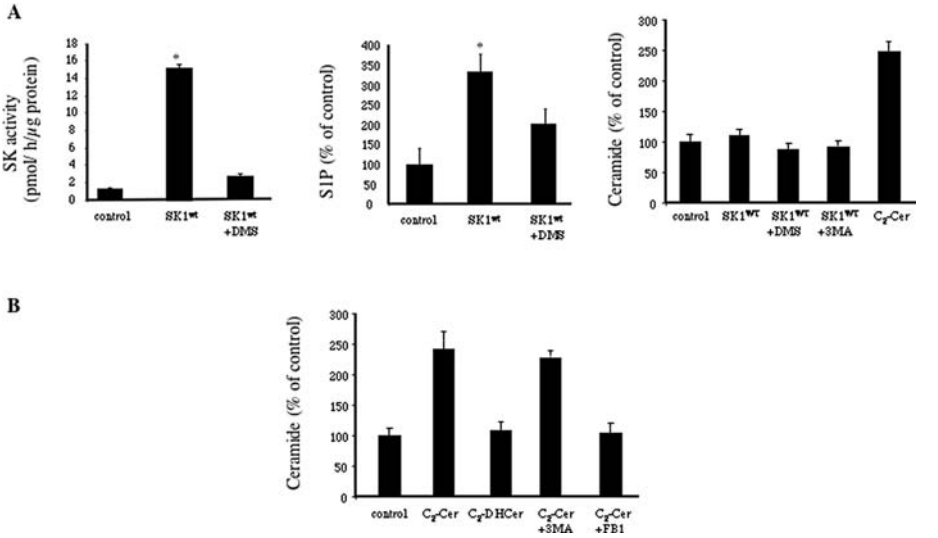

Fig. 2. Sphingolipid level and sphingosine kinase activity. **(A)** MCF-7 cells were transfected with the cDNA encoding the wild-type sphingosine kinase 1 ($SK1^{wt}$) or an empty vector (control). SK activity and sphingolipid level were analyzed 24 h posttransfection. When required, cells were treated with DMS (1.5 μ*M*), FB1 (100 μ*M*), 3MA (10 m*M*). Cells were treated for 2 h with C_2-Cer (75 μ*M*). The S1P level was determined after [^{3}H]sphingosine incorporation and endogenous long-chain ceramides were quantified by the DGK assay. **(B)** Long-chain ceramide level was quantified in HT-29 cells by the DGK assay after treatment with C_2-Cer (75 μ*M*) or C_2-DHCer (75 μ*M*) for 2 h in the presence or the absence of FB1 (100 μ*M*) or 3MA (10 m*M*). (Reproduced from **refs. *5*** and ***6*** with permission from ASBMB.)

3.2.1. Lipid Extraction

1. After treatment, the cells are washed twice in ice-cold PBS, pH 7.4, scraped straight off the plate, and harvested by centrifuging at 4°C for 5 min. About one to two times 10^6 HT-29 cells (one 10-cm Petri dish, 80% confluent) can be processed using 2 mL of methanol, 1 mL of chloroform, and 0.6 mL of water. After centrifuging, the solvents are added to dry pellets of cells, in 13 × 100 mm glass screw-cap test tubes, and the capped tubes are then vortexed immediately for 15 s (*see* **Note 4**).
2. The samples are kept at room temperature for at least 10 min and are then vortexed again for 15 s. Chloroform (1 mL) and water (1 mL) are then added, and samples are vortexed once more for 15 s. This results in a biphasic mixture. The biphasic mixture is composed of a lower lipid-containing chloroform phase and an upper phase consisting of methanol and water.
3. The mixture is centrifuged at 2000*g* for 5 min at 4°C and then the two phases are separated: the organic phase (lower one) can be collected in fresh glass tubes, and

the lipids are evaporated under a stream of nitrogen or under vacuum. To provide values for phospholipids within the range of the standard values, take a 1-mL aliquot of the lower phase for ceramide determination and 0.3-mL duplicates for determining the phospholipid content (*see* **Note 5**).
4. Dry the samples for phospholipid quantification under a stream of nitrogen or under vacuum, and store the remaining sample at 20°C for the DG assay.

3.2.2. Lipid Phosphate Evaluation

1. The cellular phosphate is extracted with the lipid and is subjected to colorimetric assay, being quantified by referring to a standard curve, and provides a measurement of the cell mass of the samples. The standard measurements, ranging from 5 to 120 nmol of phosphate, are acquired by taking 5- to 120-µL aliquots of a 1 m*M* NaH_2PO_4 aqueous solution in screw-cap glass test tubes.
2. Add 0.6 mL of washing buffer to the dried lipid samples and standard tubes.
3. Put the open tubes in a heating block at 157°C for 4–16 h. Let the samples reach this temperature slowly, without preheating the block. This procedure evaporates the aqueous phase, leaving approximately 100 µL of free phosphate in the tubes.
4. Once the samples have cooled, add 0.9 mL of water, and vortex the samples for 30 s to resuspend thoroughly (*see* **Note 6**).
5. Add 0.5 mL of ammonium molybdate and vortex samples. Molybdate and phosphate will react to produce phosphomolybdic acid.
6. Add 0.2 mL of L-ascorbic acid to the samples, and mix gently. This step converts phosphomolybdic acid into molybdenum blue, which has its highest absorbance at 820 nm.
7. After incubating the samples at 45°C (water bath) for 30 min, cool for 5–10 min and read the absorbance at 820 nm in the spectrophotometer. Standard absorbances are used to plot a standard curve. The phosphate content can be determined by referring to the standard curve, allowing for the appropriate dilution factors resulting from the extraction process (*see* **Note 7**).

3.2.3. Preparation of βOG-DOPC Mixed Micelles

All glassware must be acid washed, rinsed thoroughly with water, and then rinsed with acetone and dried.

1. Take an aliquot of 0.97 mL of 20 mg/mL L-α-dioleoylphosphatidylglycerol (DOPG) in a Pyrex screw-cap tube (*see* **Note 8**).
2. Dry the DOPG as a thin film around the bottom of the glass tube (*see* **Note 9**).
3. Take up a 7.5 % solution of βOG in water, and add 1 mL to each tube containing dried DOPG.
4. Sonicate the mixture until the DOPG has completely dissolved.

Vortex vigorously and repeat the process. Store at –20°C once the micellar solution is completely clear.

3.2.4. Diacylglycerol Kinase

After lipid extraction by following the Bligh-Dyer protocol the ceramide is labelled in vitro using DG kinase ***(11,12)***, which phosphorylates both diacylglycerols and ceramides to form phosphatidic acid and ceramide-1-phosphate, respectively. This in vitro assay is precise, and makes it possible to perform a quantitative analysis of ceramide **(Fig. 2)**. To obtain the quantitative conversion of ceramide to ceramide-1-phosphate, it is crucial to perform the assay with an excess of enzyme so that the substrate is totally converted into the product ***(13,14)***. The quantification of ceramide can be determined from the slope of a standard curve using known amounts of naturally occurring ceramide and diacylglycerol after calculating the mass of ceramide-1-phosphate produced by DG kinase.

The lipids extracted are phosphorylated by the kinase, using [γ-^{32}P]ATP. The amount of enzyme per sample indicated below can be changed, depending on the activity of the protein.

1. A standard curve can be plotted using substrates of DG kinase, such as diacylglycerol and ceramide. After resuspending in chloroform, samples containing 80–2560 pmol of these standard lipids are used to plot a standard curve by taking aliquots in glass test tubes and then evaporating to dryness under a stream of nitrogen or under vacuum.
2. The extracted lipids and standards are resuspended in the mixed micelles containing a nonionic detergent and phospholipids. 20 µl of mixed micelles (*see* **Subheading 2.**) is added to the samples.
3. Vortex the lipids for 30 s, leave for 5 min at room temperature, sonicate in a water bath for 30 s and then immediately vortex again for 30 s.
4. Carefully resuspend the samples (do not vortex) in 70 µL of assay mixture containing DG kinase.
5. Adjust the volume of each sample to 70 µL using dilution buffer.
6. Begin the reaction by adding 10 µL/sample of ATP mixture.
7. Vortex the samples carefully and briefly. The reaction is then allowed to proceed for 30 min at room temperature.
8. Stop the reaction by adding 1 mL chloroform, 2 mL methanol, and 0.6 mL water. Vortex vigorously for 30 s.
9. Extract the phosphorylated lipids again according to the Bligh-Dyer procedure by adding 1 mL chloroform and 1 mL water, and vortex vigorously.
10. Separate the two phases by centrifuging at 2000*g* for 5 min at 4°C.
11. Discard the upper phase that contains about 95% of the radioactivity. Aliquot the lower phase, containing the phosphorylated lipids, into fresh tubes and dry under a stream of nitrogen or under vacuum.

3.2.5. Separation of Reaction Products by TLC

Various substrates present in lipid extracts are commonly phosphorylated by DG kinase. This means that liquid scintillation counting of the organic extract

obtained after the DG kinase reaction does not provide an exact determination of the ceramide 1-[^{32}P]phosphate content. The products of the DG kinase reaction are therefore subjected to TLC, and the ceramide-1[^{32}P]phosphate is identified by its co-migration with phosphorylated ceramide standards and on the basis of its R_f value.

1. Resuspend dried lipids in 80 µL of a mixture of chloroform-methanol (1:1, by vol.) and vortex in capped tubes for 30 s.
2. Immediately afterwards, apply 20 µL at the origin of a prescored silica gel 60 plate (*see* **Note 10**).
3. Use an appropriate solvent system to separate lipids by migration on the silica. Place the silica plate in the chamber (inside the paper ring), and allow the solvent front to migrate to the top of the plate (*see* **Note 11**).
4. After marking the solvent front lane, dry the plate in a chemical hood for 15–30 min before exposing to an x-ray film (16–24 h of exposure at –80°C is usually necessary).
5. Spots of interest are scraped from the plate and quantified by liquid scintillation counting (*see* **Note 12**). Diacylglycerols and short-chain ceramides will appear as their phosphorylated derivatives, with R_f values of 0.62 and 0.25–0.3, respectively.
6. The values of the radioactive counts are normalized for the concentrations of total phospholipids in the samples.

3.3. Sphingosine Kinase Activity and Sphingosine 1-Phosphate Production

Phosphorylation of sphingosine can be catalyzed by two sphingosine kinases, SPHK1 and SPHK2, the enzymatic properties of which exhibit some differences ***(15,16)***. To obtain reliable and reproducible estimation of the kinase activity, the activities of S1P lyase and phosphatases have to be blocked; this is accomplished by adding the required inhibitors to the sphingosine kinase buffer. The assay for sphingosine kinase activity presented here is essentially that developed by Spiegel and coworkers ***(17,18)*** **(Fig. 2)**. In this assay, sphingosine is converted by sphingosine kinase into radiolabeled S1P in the presence of [γ-^{32}P]ATP. The radiolabeled S1P is then separated from the substrates by solvent extraction and TLC. In this procedure, which uses acidic conditions, most of the S1P is extracted in the organic phase.

To determine the amount of S1P produced **(Fig. 2)**, we propose an assay based on the ability of intact living cells to phosphorylate an exogenously administered radioactive sphingoid base ([3-^{3}H]sphingosine). The radiolabeled S1P formed by the cells is then extracted from the cells or culture medium (S1P has amphiphilic properties, which make it likely that it will cross cell membranes and be secreted into the extracellular medium) using organic

solvents. Provided that the lipid extraction is carried out under alkaline conditions, S1P can be recovered from the aqueous phase ***(19)***.

3.3.1. Preparation of Samples for the Assay of Sphingosine Kinase Activity

1. Preparation of cell lysates: Control MCF7 cells or SK1-overexpressing MCF-7 cells (5×10^5 cells/well in 6-well plates) are incubated and exposed to the appropriate stimulus. At the end of the incubation time, wash the cells twice with PBS. Scrape the cells with a rubber policeman and lyse in sphingosine kinase buffer. Centrifuge cell lysates at 100,000*g* for 90 min at 4°C (using a Beckman Ti50 rotor). Collect the supernatants. Determine the protein concentration of aliquots using the technique described by Bradford ***(20)***, and the Bio-Rad protein assay reagent. Store supernatants at –80°C.
2. Preparation of sphingosine-Triton X-100 micelles: prepare a 1 m*M* solution of sphingosine containing 5% Triton X-100 by mixing 5 µL of the sphingosine stock solution and 245 µL of 5% Triton X-100 and sonicating for 5 min in a water bath.
3. Preparation of the ATP mixture: just before the enzyme assay, prepare a solution that contains 20-m*M* unlabeled ATP and 200-m*M* $MgCl_2$. Since each assay sample requires 10 µL of the mixture, to 9 µL of this solution add 1 µL of a [γ-^{32}P]ATP solution at approximately 10 µCi/µL. Take an aliquot of this mixture and count in a scintillation counter.

3.3.2. Assay of Sphingosine Kinase Activity on Cell Lysates

1. Place glass test tubes on a rack in ice.
2. Add cytosolic extract (50–200 µg of protein) or, for the blank, sphingosine kinase buffer. Then, add sphingosine kinase buffer to obtain a volume of 180 µL. Add 10 µL of the 1 m*M* sphingosine-Triton X-100 micelles (*see* **Note 13**).
3. Start the reaction by adding 10 µL of the [γ-^{32}P]ATP solution.
4. Incubate for 30 min at 37°C in a shaking water bath.
5. Transfer the tubes into ice. Stop the reaction by adding 20 µL 1 *N* HCl and 0.8 mL of chloroform/methanol/12 *N* HCl (100:200:1, by vol.). Mix thoroughly, and allow to stand at room temperature. Five minutes later, add 0.24 mL chloroform and 0.24 mL 2 *N* KCl. Vortex again and, 5 min later, centrifuge at 1000*g* for 10 min.
6. Aspirate 0.4 mL of the lower organic phase, and transfer into a fresh test tube. Add 0.9 mL of the theoretical upper phase, and mix vigorously. Five minutes later, centrifuge at 1000*g* for 10 min.
7. Aspirate 0.4 mL of the lower organic phase, and dry under a stream of nitrogen.
8. Dissolve in 60 µL of chloroform/methanol (2:1, by vol.), and spot 30 µL onto a TLC plate. Samples should be applied 2 cm from the bottom of the plate. Also spot an S1P standard on a separate lane. Applications can be made by spotting 5-µL aliquots and then drying with a hair dryer.

9. Place the TLC plate in the TLC tank, put the lid on, and then let the solvent migrate until it reaches about 1–2 cm from the top of the plate. Remove the plate and let dry in a hood.
10. Expose the plate to iodine vapors by putting it in a TLC chamber containing iodine. Once the lipids are visualized, take a picture of the plate or mark the area corresponding to the S1P standard (with a pencil). Then let the iodine evaporate by leaving the plate in a hood (a warm hair dryer can be used to accelerate the evaporation of the iodine).
11. Wrap the plate in plastic (Saran) film, and expose to autoradiography film. After overnight exposure, develop the film. Mark the areas corresponding to the radiolabeled S1P, and then scrape these areas into a piece of paper. Transfer the silica to scintillation vials, add scintillation fluid, shake, and count in a scintillation counter.
12. Calculate the sphingosine kinase activity (expressed as pmol/min/mg) in each sample as follows: first calculate the number of cpm per pmol of total ATP (*see* **Note 14**). Assuming that the S1P formed has the same specific radioactivity as ATP, convert the number of cpm scraped off the plate (after subtracting the blank) into pmol of S1P. Correct for the incubation time, the quantity of protein, and the volume of sample spotted onto the TLC plate.

3.3.3. Determination of S1P Production by Intact Cells

1. Control MCF7 cells or SK1-overexpressing MCF-7 cells (10^5 cells) are plated in 25-cm^2 cell culture flasks.
2. Twenty-four hours later, when confluence has almost been reached, the culture medium is replaced by fresh FBS-free medium containing 0.3 μCi/mL of [3-^{3}H]sphingosine (as an ethanolic solution). Any compound of interest to be tested is then added.
3. After incubating at 37°C for the desired time (from 3 to 24 h), the culture medium is collected and frozen at –20°C. The cells are washed twice with PBS, and scraped off with a rubber policeman. Sedimented cells are then frozen at –20°C.
4. The cell pellets are resuspended in 200 μL distilled water and lysed by freeze-thawing. An aliquot can be kept to determine the protein content.
5. Add 1.5 mL of chloroform/methanol (1:2, by vol) to cell lysates or aliquots (0.2 mL) of culture medium, mix, and then add 0.7 mL of 0.5 *N* NaOH followed by 1 mL chloroform to induce phase separation (*see* **Note 15**).
6. After vortexing thoroughly and centrifuging (at 1000*g* for 15 min), a 1-mL aliquot of the upper phase is aspirated for liquid scintillation counting.

4. Notes

1. Amino acids are physiological inhibitors of autophagy ***(21)***. The choice of amino acid used during the pulse chase is important, because some of them, such as leucine, are potent inhibitors of autophagy. Valine is frequently used because

this amino acid does not interfere with autophagy in most cell types. A cocktail of amino acids (with the following final concentrations of amino acids (in μM: asparagine: 60; isoleucine: 100; leucine: 250; lysine: 300; methionine: 40; phenylalanine: 50; proline: 100; threonine: 180; tryptophan: 70; valine: 180; alanine: 400; aspartate: 30; glutamate: 100; glutamine: 350; glycine: 300; cysteine: 60; histidine: 60; serine: 200; tyrosine: 75; ornithine: 100 corresponding to concentrations four times greater than those in the portal vein of starved rats) also very potently inhibits autophagy.

2. HBSS is used when the cells are incubated in a humidified chamber at 37°C in the absence of CO_2; otherwise EBSS is used.
3. 3-MA blocks autophagy by inhibiting class III phosphatidylinositol 3-kinase ***(22)***. However, it should be kept in mind that 3-MA is a phosphatidylinositol 3-kinase inhibitor ***(23)*****,** which interferes with other intracellular trafficking pathways dependent on phosphatidylinositol 3-kinases ***(24)***. 3-MA also affects some other intracellular events ***(25)***.
4. Cell pellets, resuspended in methanol, can be stored for up to 15 d at –80°C.
5. Dried lipids can be stored for up to 30 d at –80°C.
6. Decanting water around the tube walls will make it easier to collect of the residues of samples spread by fumes during heating.
7. Approximately 1×10^6 HT-29 cells correspond to 30–40 nmol of lipid phosphate.
8. The DOPG stock solution should contain 20 mg/mL or 27 m*M* of DOPG in chloroform.
9. If the DOPG is dried as a pellet at the bottom of the tube, it is more difficult to dissolve as an aqueous solution.
10. To resuspend all the lipids contained in the tubes, take a sufficient volume of solvent. When taking 20-μL aliquots out of 80 μL, it is crucial not to let the solvents evaporate since this would modify the concentration of lipids. The remaining lipids can be dried and saved for another run, if this is required. It is necessary to preclean the silica plate in acetone for 2 h before use, and let it dry completely before applying the samples.
11. Prepare a well-sealed chamber at least 12 h before use to achieve equilibrium between liquid-vapor phases of the solvent mixture. Since saturating conditions are important during the run, it is essential to leave the tank open as briefly as possible while inserting the plate.
12. Radioactivity associated with ceramide 1-^{32}phosphate is represented by two spots with R_f values of 0.47–0.41 corresponding to ceramides with different acyl chain length and/or dihydroceramides.
13. Determining sphingosine kinase activity in different concentrations of Triton X-100 may makes it possible to discriminate between the activities of SPHK1 and SPHK2. High concentrations (e.g., 0.5%) of Triton X-100 have been reported to inhibit SPHK2 activity, whereas they stimulate SPHK1 activity ***(16)***.
14. The range of protein concentrations giving a linear response for enzyme activity is cell type–dependent.

15. The extraction of S1P from cell lysates or culture medium and their separation from sphingosine can be performed according to the procedure reported by Vessey et al., which also uses alkaline conditions ***(26)***.

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