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Determination of sphingosine-1-phosphate lyase activity by gas chromatography coupled to electron impact mass spectrometry

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ABSTRACT

Sphingosine-1-phosphate lyase (SGPL1) is the last enzyme in the catabolism of sphingolipids. It catalyzes the retroaldolic cleavage of long chain base phosphates into phosphoethanolamine and a fatty aldehyde. In this article we report on an easy and sensitive procedure to determine SPL activity. The assays uses C17-sphinganine-1-phosphate as substrate and the aldehyde product, pentadecanal, is quantified as its pentafluorobenzyloxime derivative by GC/MS. Derivatization of pentadecanal is performed as a one-step reaction, and the oxime product is directly injected for GC/MS analysis without any further purification. Acquisition in selected ion monitoring mode allows very high sensitivity, with a limit of detection of 281 fmol. The assay is linear with both protein concentration and incubation time up to 20 μg and 40 min, respectively. The K_m value obtained (6 μM) is similar to that for the natural substrate sphingosine-1-phosphate. Using this method, FTY720 and deoxypyridoxine phosphate inhibited SPL with similar potencies to those reported.

1. Introduction

The bioactive lipid sphingosine-1-phosphate (S1P) and its saturated analog sphinganine-1-phosphate (dhS1P) are catabolized to ethanolamine phosphate (EAP) and (*E*)-2-hexadecenal or hexadecanal, respectively (Ikeda et al., 2004; Van Veldhoven and Mannaerts, 1991, 1993). This reaction is accomplished by the pyridoxal 5′-phosphate dependent enzyme sphingosine-1-phosphate lyase (SGPL1), encoded by the *SGPL1* gene. This enzyme serves central roles in development (Herr et al., 2003; Kihara et al., 2003; Li et al., 2001) and chemotaxis (Kumar et al., 2004), it prevents defects in reproductive structures and function (Phan et al., 2007), acts as a tumor suppressor (Oskouian et al., 2006; Reiss et al., 2004), has a role in chemoresistance (Alexander et al., 2006; Li et al., 2000; Min et al., 2004, 2005) and it is also implicated in immunity (Schwab et al., 2005). Therefore, SGPL1 has emerged as a novel therapeutic target in cancer and immunosuppression (Bandhuvula and Saba, 2007; Ling et al., 2009; Oskouian and Saba, 2010) and the identification of compounds able to modify SGPL1 activity has attracted interest in a therapeutic context.

SGPL1 activity can be determined using radioactive, fluorescent and fluorogenic substrates. The first one involves incubation of the enzyme with [4,5-^{3}H] dihydrosphingosine-1-phosphate and, after extraction of lipids into an organic phase under acidic conditions followed by thin-layer chromatography, the radioactive aldehyde is quantified by liquid-scintillation counting, autoradiography or phosphorimaging (Van Veldhoven, 2000; Van Veldhoven and Mannaerts, 1991). In the case of fluorescent substrates, the resulting aldehyde is also extracted from the incubation mixture and quantified after separation by HPLC coupled to a fluorescent detector (Bandhuvula et al., 2007, 2009). In a recent assay, a non fluorescent coumarinic substrate is cleaved by SGPL1 to release an aldehyde which undergoes a spontaneous β-elimination reaction to give umbelliferone. No separation of products is necessary and the assay can be performed in microtiter wells, which is an important improvement in high throughput screening of putative inhibitors (Bedia et al., 2009). Nevertheless, given the high K_m of SGPL1 for the fluorogenic substrate (152 μM), kinetic characterization of inhibitors should be ideally carried out with a minimally modified alternative substrate. In this article we report on a new assay for SGPL1 using C17-sphinganine-1-phosphate (C17-dhS1P).

Abbreviations: C15-AL, pentadecanal; C15-PFBO, pentadecanal *O*-(2,3,4,5,6-pentafluorobenzyl) oxime; C17-dhS1P, C17 dihydrosphingosine-1-phosphate; dhS1P, dihydrosphingosine-1-phosphate; DL, detection limit; GC/MS, gas chromatography; DOP, deoxypyridoxine phosphate; MEF, mouse embryonic fibroblasts; MS, mass spectrometry; PFBHA, *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride; PFBO, *O*-(2,3,4,5,6-pentafluorobenzyl) oxime; S1P, sphingosine-1-phosphate; S1P-C18NBD, ω (7-nitro-2,1,3-benzoxadiazol-4-yl)-D-*erythro*-sphingosine-1-phosphate; SGPL1, sphingosine-1-phosphate lyase; SIM, selected ion monitoring; Z11C16-PFBO, (*Z*)-11-hexadecenal *O*-(2,3,4,5,6-pentafluorobenzyl) oxime; Z11C16-AL, (*Z*)-11-hexadecenal.

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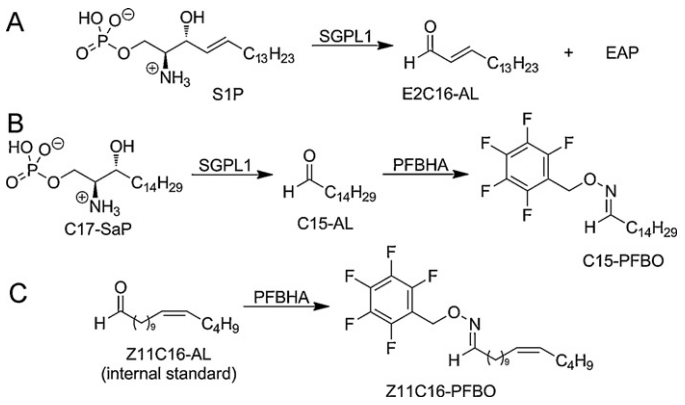

Fig. 1. SGPL1 reaction on (A), the natural substrates, S1P and dhS1P and (B), C17-dhS1P. The perfluorobenzyloximes (PFBO) produced upon derivatization of C15-AL (analyte) and Z11-C16-AL (internal standard) are also shown in B and C, respectively.

The released pentadecanal (C15-AL) is easily measured by gas chromatography coupled to mass spectrometry (GC/MS) in selected-ion acquisition mode after derivatization with pentafluorobenzylhydroxylamine (PFBHA) (Fig. 1). The use of C17-dhS1P as substrate limits possible interference by endogenous fatty aldehydes, either free or inadvertently generated from plasmalogens. Thus, while these aldehydes generally contain an even number of carbon atoms (Ingrand et al., 2000), SGPL1 cleavage of C17-dhS1P affords C15-AL, with an odd number of carbon atoms and unambiguously distinguishable from natural aldehydes.

2. Material and methods

2.1. Reagents

The substrate C17-dhS1P was purchased from Avanti Polar Lipids; C15-AL, (*Z*)-11-hexadecenal (Z11C16-AL), PFBHA hydrochloride (PFBHA HCl) and Dulbecco's modified essential medium (DMEM) were from Sigma–Aldrich, and FTY720 was from Cayman Chemical Company and fetal bovine serum (FBS) was from Invitrogen.

2.2. Cell culture

Mouse embryonic fibroblasts (MEF) (Van Veldhoven, 2005) are cultured in DMEM supplemented with 10% FBS, at 37 °C and 5% CO_2.

To prepare cells at different confluences, different volumes (1.5, 2, 3 and 4 mL) of a cell suspension (3.3×10^6 cells/mL) prepared from a 175 cm^2 confluent flask of cells were added to 175 cm^2 flasks and cells were incubated for 24 h. After this time, cells were observed under an inverted microscope and confluence was determined visually. Cells were then detached (trypsin–EDTA), washed (PBS), counted and diluted with PBS to the required density. Unless specified otherwise, all the experiments were carried out at 80–90% confluence.

2.3. Preparation of calibration curves

To prepare stock solutions, Z11C16-AL and C15-AL were dissolved in hexane at 10 mM and stored at 4 °C. To construct the calibration curves, the stock solution of C15-AL was diluted 50-fold to obtain a concentration of 200 μM, which was then submitted to serial dilutions (1/2) to obtain a concentration range of 200–0.2 μM. To 5 μL of each dilution was added the internal standard, Z11C16-AL (5 μL, 20 μM final concentration), samples were carefully evaporated under a stream of nitrogen to almost dryness and aldehydes were derivatized to their corresponding pentafluorobenzyloximes (PFBO) with 50 mM PFBHA HCl in 50 mM Tris–HCl buffer, pH 7.4 (100 μL). The solutions were incubated at 37 °C for 15 min and extracted with one volume of hexane. The solvent was evaporated under a stream of nitrogen. Residues were solubilized in 10 μL of hexane and stored at −20 °C until GC/MS analysis.

2.4. Standard SGPL1 assay

The assay was carried out by sequentially adding to the reaction buffer (125 μL per replicate) the solutions of both substrate (25 μL, 32 μM, 8 nmol per replicate) and protein (50 μL per replicate; 0.4 mg/mL of protein). The reaction buffer was prepared as follows: to 150 μL of 0.5 M potassium phosphate buffer pH 7.4 was added to the following solutions in the same buffer: pyridoxal phosphate (2 μL, 12.5 mM), sodium orthovanadate (2 μL, 1.25 mM), EDTA (2 μL, 250 mM), sodium fluoride (2 μL, 1.25 mM) and DTT (2 μL, 100 mM). To prepare the substrate solution, 40 μL of a 0.2 mM C17-dhS1P solution in ethanol (8 nmol) was added to an eppendorf tube and the solvent was removed under a stream of nitrogen. To the residue was added 25 μL of a 1% solution of Triton-X 100 in milliQ water and the mixture was sonicated for 1 min in an ultrasound bath. To obtain the protein solution, cells, collected by trypsinization, were washed with PBS and then suspended in 0.5 M potassium phosphate buffer pH 7.4 (0.4 mg/mL of protein) and the mixture was sonicated for 10 min in ice-cold water in an ultrasound bath.

After incubation for 40 min at 37 °C, Z11C16-AL (5 μL, 25 μM in ethanol, 125 pmol) and PFBHA (250 μL, 50 mM in methanol, 12.5 μmol) were sequentially added and the mixture was incubated at 37 °C for 30 min after vigorous stirring. The PFBO were extracted with hexane (500 μL) and 400 μL of the organic solution was stored at −20 °C until analysis. Before injection, the solvent was carefully removed (nitrogen stream), 10 μL of hexane were added and 2 μL were injected into the GC/MS equipment for analysis. Concentrations of the product, C15-AL, were calculated from the area ratios between both C15-PFBO and Z11C16-PFBO in reference to a calibration curve.

2.5. Determination of the recovery and the detection limit

Different volumes of a C15-AL ethanol solution to afford a range of 10, 7.5, 5, 2.5 and 1 pmol were mixed with Z11C16-AL (10 pmol) and then 200 μL of the assay reaction buffer (see below) containing 20 μg of boiled cell lysate was added, followed by addition of 50 mM PFBHA HCl in 50 mM Tris–HCl buffer, pH 7.4 (100 μL). The solutions were vigorously stirred and extracted with one volume of hexane. The solvent was evaporated under a stream of nitrogen. Residues were solubilized in 10 μL of hexane and 2 μL were injected into the GC/MS instrument. Chromatographic conditions and acquisition mode are detailed below. The amount of C15-AL whose PFBO derivative gives a signal-to-noise ratio of 2 was set as the detection limit. To determine the recovery, the same amounts of C15:PFBO were injected and the areas were compared with those of the above fortified samples.

2.6. Pentadecanal stability

To 125 μL of the reaction buffer was added 50 μL of protein extract (0.4 mg/mL, 20 μg) and 40 μL of a 0.5 μM ethanol solution of pentadecanal (20 pmol). After incubation at 37 °C for 1 h, Z11C16-AL (10 μL, 25 μM in DMSO, 250 pmol) and PFBHA (250 μL, 50 mM in Tris–HCl buffer pH 7.4, 12.5 μmol) were sequentially added and the mixture was incubated at 37 °C for 15 min after vigorous stirring. The resulting PFBO were extracted and analyzed as described above.