RESEARCH COMMUNICATION

Sphinganine 1-phosphate metabolism in cultured skin fibroblasts: evidence for the existence of a sphingosine phosphatase

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On addition of [4,5-³H]sphinganine 1-phosphate to human fibroblast monolayers, the label was efficiently removed from the culture medium. In contrast with the reported stability of phosphorylated sphingenine in 3T3 cells [Desai, Zhang, Olivera, Mattie and Spiegel (1992) *J. Biol. Chem.* **267**, 23122–23128] and B16 melanoma cells [Sadahira, Ruan, Hakomuri and Igarashi (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9686–9690], sphinganine 1-phosphate appeared to be subjected to a fast and extensive metabolism in fibroblasts, the major pathways being cleavage and dephosphorylation. The first of these pathways, catalysed by

sphingosine-phosphate lyase, resulted in the formation of labelled palmitaldehyde, which was recovered, mainly after oxidation, in glycerophospholipids in an ester bond. A smaller part of the palmitaldehyde was reduced and incorporated in alk(en)yl-phospholipids. Dephosphorylation of sphinganine 1-phosphate, a hitherto overlooked pathway catalysed by an unknown phosphatase(s), gave rise to sphinganine, which was converted by N-acylation into ceramide and then incorporated in sphingomyelin and glycosphingolipids.

INTRODUCTION

Sphingolipids are degraded in the lysosomes to their basic constituents, a sphingoid base, a fatty acid and a polar headgroup [1]. [The term sphingoid base refers to a group of related aliphatic 2-amino-1,3-diol compounds, the most prevalent of which are sphinganine (2*D*-amino-1,3*D*-octadecanediol) and sphingenine (4*E*,2*D*-amino-1,3*D*-octadecanediol). The generic term sphingosine is used to allude to C₁₈ sphingoid bases without specifying their substituents or degree of unsaturation, but possessing the (2*D*,3*D*) configuration.] The sphingoid base, after phosphorylation by sphingosine kinase [2], is then cleaved by sphingosine-phosphate lyase, to a fatty aldehyde and phosphoethanolamine [3]. Phosphorylated sphingoid bases not only appear to be intermediary catabolites, but are also endowed with bioactivity. Sphingenine 1-phosphate given to cultured 3T3 cells mediates Ca²⁺ release from intracellular stores and stimulates DNA synthesis in quiescent cells [4]. The reported mitogenicity of sphingenine in these cells [5] thus appeared to be due to its phosphorylated metabolite. In a subsequent study, however, Spiegel and co-workers [6] demonstrated that sphingenine 1-phosphate itself was not mitogenic, but was responsible for an increase in phosphatidate levels, apparently through stimulation of a phospholipase D. Phosphatidate is known to be a potent mitogen for various cells, and phospholipase D activation occurs in many transduction processes (see [7,8]). Another effect of sphingenine 1-phosphate which has been observed in B16 melanoma cells is the inhibition of cell motility and invasiveness, through an as yet unknown mechanism [9]. Hence it is conceivable that sphingosine kinase and sphingosine-phosphate lyase are not merely involved in the catabolism of sphingolipids, but could also produce and attenuate those intermediates interacting with the phospholipase D and/or Ca²⁺-dependent signalling pathways. Although this is an attractive hypothesis, modulation of intracellular levels of phosphorylated sphingoid bases in response to physiological stimuli remains to be proven. Moreover, our

knowledge of the metabolic fate of sphingoid bases and their phosphorylated products is rather poor, as demonstrated by the initial confusing data obtained in sphingenine-treated 3T3 cells are reported above. Sphingenine 1-phosphate, taken up by 3T3 cells [4,6] or generated in sphingenine-supplemented 3T3 cells [4] or B16 cells [9], appears to be metabolically stable. This is in contradiction with our own data showing sphingosine-phosphate lyase and sphingosine-phosphatase(s) activities in homogenates of different cultured cells such as skin fibroblasts, 3T3-L₁ and Swiss 3T3 cells (see [3]). The action of either of these enzymes would lead to an attenuation of sphingenine 1-phosphate. Therefore, a study was initiated on the metabolism of [4,5-³H]sphinganine 1-phosphate in cultured fibroblasts.

MATERIALS AND METHODS

Materials

1-*O*-Alkyl-glycerophosphocholine (lyso-platelet activating factor), ceramides (*N*-palmitoyl-*D*-sphingosine and type IV, containing *N*- α -hydroxy fatty acids), galactocerebrosides (type I and type II), lactoside, L- α -phosphatidylethanolamine (type I-A, containing approx. 60% plasmalogen), phospholipase D (*Streptomyces chromofuscus*; 3000 units/mg of protein), psychosine, sphinganine (*D,L*-dihydrosphingosine), sphingenine (*D*-sphingosine), sphingenylphosphocholine (sphingosylphosphorylcholine), sphingomyelin (egg yolk), sulphatide and other lipids were obtained from Sigma, St. Louis, MO, U.S.A. Amplify spray was from Amersham, Bucks., U.K. and En^hance spray from New England Nuclear, Bad Homburg, Germany. Methylamine [33% (w/v) in ethanol] was purchased from Fluka, Buchs, Switzerland.

Preparation of lipids and t.l.c. analysis

Palmitaldehyde, sphinganylphosphorylcholine, sphinganine 1-phosphate, sphingenine 1-phosphate and [4,5-³H]sphinganine

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Table 1 Mobilities of standards lipids

Standards, applied on silica G 60A plates, were chromatographed in the solvent systems described in the Materials and methods section. After visualization, the R_F values were determined. Abbreviation: n.d., not determined.

Standard	Solvent system...	R_F value						
		B	C	D	E	G	F	I
Fatty alcohol		0.55	0.62	0.94	0.88	0.92	n.d.	0.97
Fatty aldehyde		0.70	0.72	0.95	n.d.	0.92	n.d.	0.99
Fatty acid		0.53	0.04	0.50	0.87	0.88	0.83	0.98
<i>N</i> -Methyloleoylamide		0.48	0.66	0.95	0.88	0.91	0.77	0.94
1-Acyl-glycerophosphate		0.00	0.00	0.02	0.29	0.30	0.54	0.50
Phosphatidic acid		0.00	0.00	0.05	0.58	0.44	0.64	0.80
Cardiolipin		0.00	0.00	0.54	0.70	0.57	0.64	0.92
1-Acyl-glycerophosphocholine		0.00	0.00	0.08	0.05	0.27	0.16	0.11
1-Alkyl-glycerophosphocholine		0.00	0.00	0.08	0.042	0.27	0.16	0.12
Phosphatidylcholine		0.00	0.00	0.26	0.13	0.40	0.40	0.35
1-Acyl-glycerophosphoethanolamine		0.00	0.00	0.20	0.29	0.40	0.47	0.29
1-Alk(en)yl-glycerophosphoethanolamine		0.00	0.00	0.20	0.24	0.40	0.50	0.36
Phosphatidylethanolamine		0.00	0.00	0.45	0.59	0.66	0.59	0.62
Phosphatidylserine		0.00	0.00	0.08	0.25	0.38	0.54	0.54–0.60
Phosphatidylinositol		0.00	0.00	0.09	0.40	0.35	0.53	0.35
Sphinganine		0.005	0.10	0.74	0.58	0.34	0.64	0.67
Sphinganine		0.01	0.14	0.80	0.59	0.39	0.65	0.68
Ceramide		0.31	0.48	0.95	0.92	0.92	0.83	0.98
Ceramide (<i>N</i> -hydroxy fatty acid)		0.22	0.30	0.95	0.92	0.90	0.83	0.98
Psychosine		0.00	0.00	0.28	0.29	0.25	0.52	0.34
Cerebroside (type I)		0.015	0.02	0.51	0.81	0.64	0.65	0.77
Cerebroside (type II)		0.025	0.03	0.59	0.84	0.69	0.68	0.83
Lactosylceramide		0.00	0.00	0.23	0.70	0.53	0.59	0.55
Sulphatide		0.00	0.00	0.54	0.77	0.47	0.64	0.65
Sphinganine 1-phosphate		0.00	0.00	0.01	0.12	0.23	0.50	0.30
Sphinganine 1-phosphate		0.00	0.00	0.01	0.13	0.25	0.51	0.31
Ceramide 1-phosphate		0.00	0.00	0.05	0.46	0.42	0.56	0.69
Sphinganylphosphocholine		0.00	0.00	0.06	0.014	0.08	0.14	0.05
Sphingenyphosphocholine		0.00	0.00	0.06	0.014	0.09	0.14	0.05
Sphingomyelin		0.00	0.00	0.13–0.16	0.09	0.33–0.38	0.18–0.35	0.21–0.32

1-phosphate were prepared as described previously [10]. Ceramide 1-phosphate was obtained by phospholipase D treatment of sphingomyelin as described in [11] with some modifications.

N-Methyloleoylamide was prepared by transesterification of triolein [200 mg in 0.2 ml of *n*-butanol/0.5 ml of 33 % (w/v) methylamine in ethanol] for 1 h at 50 °C (modified from [12]). After adding 4 vol. of water, the product was extracted in diethyl ether, dried and stored in chloroform at –20 °C.

1-Alk(en)yl-glycerophosphoethanolamine was prepared by hydrolysing phosphatidylethanolamine, enriched in ether-linked lipids [25 mg in 0.1 ml of *n*-butanol plus 0.4 ml of 33 % (w/v) methylamine in ethanol], during incubation for 90 min at 70 °C [12,13]. After adding 1.6 ml of 1 M NaCl, the *N*-methyl fatty amides generated were extracted into diethyl ether. The aqueous phase, containing the alkali-resistant 1-alk(en)yl-glycerophosphoethanolamine, was brought to pH 4 with acetic acid and phase-separated according to Bligh and Dyer [14]. Approx. 90 % of the final product appeared to contain an acid-labile vinyl ether bond.

T.l.c. was performed on silica G 60A plates (0.25 mm thick) (Merck, Darmstadt, Germany). The compositions of the different solvent systems used in this study were as follows: A, hexane/diethyl ether/acetic acid (70:30:1, by vol.); B, chloroform/methanol/acetic acid (95:5:1, by vol.); C, chloroform/methanol/25 % ammonia (90:10:1, by vol.); D, chloroform/methanol/25 % ammonia (12:6:1, by vol.); E, chloroform/methanol/acetic acid/water (65:43:1:3, by vol.); F, *n*-butanol/acetic acid/water (3:1:1); G, chloroform/

methanol/water (60:35:8, by vol.); H, chloroform/methanol/acetic acid/water (25:15:4:2, by vol.), and I, chloroform/methanol/formic acid (13:5:2, by vol.). Mobilities of standards obtained in some of these systems are given in Table 1. Lipids were visualized with iodine or by specific staining [15]. To detect the incorporation of label into the different lipids, the dried t.l.c. plates were sprayed with En^hance or Amplify and exposed to Kodak XAR-5 films at –80 °C. Spots were identified by comparing their R_F values with those of suitable standards, spotted on the same plate, as well as on the basis of their sensitivity to different hydrolytic treatments or their known behaviour during phase separations. Lipids were scraped into 0.5 ml of 1 % (w/v) SDS and radioactivity was measured after adding 4 ml of Instagel II. If appropriate, values were corrected for experimentally determined extraction efficiencies (see below), but not for the possible lower counting efficiencies of tritiated lipids adsorbed to silica. Using the described methodology, no differences in counts were seen for tritiated palmitic acid, sphinganine and sphingomyelin in solution or adsorbed to silica gel.

Cell cultures

Skin fibroblasts from healthy individuals or children affected with X-linked adrenoleukodystrophy or with Zellweger syndrome were obtained through the courtesy of the Center for Human Genetics of our University. Fibroblasts were cultured as described previously [15].

Sphinganine 1-phosphate metabolism

For the experiments on uptake and metabolism of sphinganine 1-phosphate, control fibroblasts were seeded in 60-mm-diam. dishes and grown until confluent. The medium was then replaced with 2.5 ml of medium containing 1 μ M sphinganine 1-phosphate, which was prepared as follows. An aliquot of 0.5 mM sphinganine 1-phosphate (a sonicated solution in ethanol; specific radioactivity 40 μ Ci/ μ mol) was added aseptically to 4 vol. of 0.6% (w/v) defatted albumin (dissolved in physiological salt solution and sterilized by means of filtration). After incubation at 37 °C for 1 h to promote binding of the lipid to albumin, this solution was diluted 100-fold in Hepes-fortified culture medium [Dulbecco's modified Eagle's medium/Ham's F12 (1:1, v/v)] containing 0.2% Ultrosor G.

At the indicated time points, the medium was removed, the monolayer was washed twice with cold PBS and fixed with 1.1 ml of methanol [16]. The cells in methanol were scraped and transferred to extraction tubes. The dishes were washed once with 1.1 ml of methanol, which was then combined with the first methanol solution, followed by the addition of 1 ml of chloroform. After extracting the cellular lipids for 1 h, insoluble residue was removed by centrifugation and re-extracted with 3 ml of chloroform/methanol (1:2, v/v). After centrifugation, both organic extracts were combined (6 ml).

In order to investigate the incorporation of label in neutral lipids and in polar lipids, either present in the O-ester or a vinyl ether bond, half of the lipid extract was subjected to phase separation under neutral conditions by adding 1 ml of chloroform and 1.8 ml of 0.5 M NaCl. Aliquots of the lower phase (2.15 ml of total volume) were dried under N_2 , dissolved in chloroform/methanol (9:1, v/v) and separated by t.l.c. in systems A, B and C. To detect O-esterification, samples were analysed with or without preceding alkaline hydrolysis using methylamine [13]. To liberate the fatty aldehydes linked via vinyl ether bonds, spotted lipid extracts were exposed to HCl fumes before chromatography.

To investigate the labelling of sphingolipids, 2 ml of the initial lipid extract was dried, dissolved in 1 ml of 0.2 M NaOH in methanol/water (9:1, v/v) and hydrolysed at 37 °C for 1 h [17]. Phase separation was obtained by adding 2 ml of chloroform, 1.1 ml of methanol, 1 ml of 1 M NaCl and 0.7 ml of 0.5 M NaOH. The upper phase was transferred to another tube, together with the upper phase obtained after an additional wash with 2 ml of synthetic upper phase. By adding different sphingolipid and phospholipid standards to mock extractions, it was found that sphinganine and sphingomyelin were quantitatively retained in the lower phase. The recovery in the lower phase of the lysocompounds, 1-alk(en)ylglycerophosphoethanolamine, sphinganylphosphocholine and 1-alkylglycerophosphocholine were lower, reaching 80, 33 and 24% respectively. Sphinganine 1-phosphate was completely recovered in the aqueous upper phase, but the generated fatty acid soaps were only partly recovered. Aliquots of the lower phase (1.85 ml) were counted and, after drying, dissolved in chloroform/methanol (4:1, v/v) and analysed by t.l.c. in system D or E. Lipids present in the alkaline upper phase were back-extracted into chloroform after acidification with 0.5 ml of 5 M HCl and addition of 4 ml of chloroform/methanol/5 M HCl (86:14:1, by vol.). The lower phase was removed and the upper phase was re-extracted once again with synthetic lower phase. After combining the two lower phases, the organic extracts were dried, dissolved in chloroform/methanol (1:1, v/v), and analysed by t.l.c. in systems F and G. Control experiments showed that the overall recoveries of 1-alk(en)ylglycerophosphoethanolamine, sphinganine 1-

phosphate, 1-alkylglycerophosphocholine and sphinganylphosphocholine reached 57, 55, 9 and 5% respectively.

In order to follow the incorporation of label into plasmalogens of fibroblasts from Zellweger and X-linked adrenoleukodystrophy patients, the lipids extracts from two 100-mm-diam. dishes, incubated for 24 h with 5 ml of sphinganine 1-phosphate-containing medium, were combined, dried and dissolved in 3 ml of chloroform/methanol (1:2, v/v) and subjected to phase separation under neutral conditions using NaCl as described above. The lower phase was dried, dissolved in chloroform/methanol (3:2, v/v) and analysed by two-dimensional t.l.c. with or without preceding alkaline hydrolysis using methylamine [13]. Lipids were separated in the first dimension in systems D or H, followed by system I in the second dimension. In between the developments, plates were exposed to HCl fumes in order to cleave the alkenyl bonds, which are present in the plasmenylcholine and -ethanolamine.

RESULTS AND DISCUSSION

For studying the uptake and metabolic conversion of phosphorylated sphinganine by skin fibroblasts, a concentration of 1 μ M was chosen. This choice was based on the concentrations reported in the literature to produce cellular effects. Maximal inhibition of motility in B16 cells is observed at 10–100 nM of sphinganine 1-phosphate [9]. Mitogenic effects on 3T3 cells become apparent at 0.5 μ M and are maximal at 2–5 μ M [4], while stimulation of phosphatidate formation is seen between 2 and 10 μ M [6]. Of course, phosphorylated sphingoid bases are amphipathic molecules, which will tend to be distributed in the membrane bilayers. Therefore, one should relate the reported effective concentrations to the number of cells used in these studies. The uptake of sphinganine 1-phosphate, in the presence of albumin, by human skin fibroblasts (determined as the disappearance of label from the incubation medium) was fast, with an initial rate of approx. 460 pmol/15 min per 10^6 cells. This is substantially higher than the value that one can calculate from experiments performed with Swiss 3T3 cells supplemented with 1 μ M [32 P]sphinganine 1-phosphate, this being 65 pmol/15 min per 10^6 cells [4]. Whether this difference is (i) due to the presence of albumin in the culture medium, (ii) related to the different position of the label (see below) or the use of saturated versus unsaturated compounds, or (iii) reflects variation between both cell lines, is not known. At a concentration of 5 μ M sphinganine 1-phosphate, uptake by Swiss 3T3 cells was augmented to 750 pmol/15 min per 10^6 cells [6].

After exposure for 20 h, approx. 75% of the label was removed from the medium by the fibroblasts (Figure 1). However, this appears to be a slight underestimation of the uptake, since analysis of the medium at that time point showed that only 70% of the radioactivity was still associated with sphinganine 1-phosphate. About 10% of the label appeared to be associated with sphinganine, whereas the remaining label was recovered in water-soluble products, most likely degradation products (water and acetic acid, see below) released from the cells. In the cell extracts the major portion of the label was not associated with sphinganine 1-phosphate, not even at the first time point after addition (15 min); this therefore indicates a fast turnover of this lipid once taken up.

As expected, one of the initial conversions of sphinganine 1-phosphate appeared to be cleavage by sphingosine-phosphate lyase to generate tritiated palmitaldehyde. Due to further metabolism, this primary product did not accumulate, but was largely oxidized to palmitic acid. After activation to palmitoyl-CoA, the fatty acid was incorporated into glycerolipids (mainly

Figure 1 Uptake and metabolism of sphinganine 1-phosphate by cultured skin fibroblasts

Fibroblast monolayers were incubated with medium containing 2.5 nmol of [4,5-³H]sphinganine 1-phosphate (220 000 d.p.m.) and processed at the indicated times as described in the Materials and methods section. Curves show the radioactivity recovered in the media (■, expressed as a percentage of the input) and associated with the lipids recovered in the cellular extracts (expressed as pmol/monolayer): ●, sphinganine 1-phosphate; △, sum of non-characterized lipids and water-soluble products; ▼, sum of palmitaldehyde and its metabolites; *, sum of sphinganine and its metabolites; □, sum of all recovered products.

Figure 2 Cellular metabolism of the cleavage products of sphinganine 1-phosphate

The radioactivity associated with cellular lipids (see Figure 1) that originate directly from the action of sphingosine-phosphate lyase (free fatty aldehyde; ■) or that are formed by further metabolism (▼, free fatty alcohol; □, free fatty acid; ▲, O-esterified fatty acids; ○, alk(en)yl-linked phospholipids; *, sum of fatty aldehyde and further metabolites) is shown.

phospholipids) (Figure 2). Some portion of the activated fatty acid is probably also degraded via mitochondrial β -oxidation. During the first cycle of β -oxidation, the tritium will appear in water or acetyl-CoA. If palmitic acid, generated intracellularly (via palmitaldehyde oxidation), is metabolized in the same way as palmitic acid taken up from the medium then the label recovered in water-soluble products would be about 4% of the label recovered in the O-esterified position ([15]; P. P. Van Veldhoven and G. P. Mannaerts, unpublished work). Reduction

of palmitaldehyde occurred as well, but was approx. 2-fold lower than its oxidation (Figure 2). In agreement with earlier studies on sphinganine metabolism by Stoffel and co-workers [18], free fatty alcohol did not accumulate, but was incorporated in ether phospholipids, mainly in plasmalogen ethanolamine and to a smaller extent in plasmalogen choline. Further desaturation to their plasmalogen form, during which half of the radioactivity is lost as water [19], appeared to be small. (In fibroblast cultures derived from patients suffering from Zellweger and X-linked adrenoleukodystrophy, two inherited metabolic peroxisomal disorders [20], the uptake and metabolism of [4,5-³H]sphinganine 1-phosphate was comparable with that seen in control cultures. As expected, no incorporation of label in (vinyl)ether lipids was observed in Zellweger syndrome. The peroxisomal enzymes, catalysing the initial steps in ether lipid synthesis [21], are deficient in this syndrome).

The other primary metabolic product of sphinganine 1-phosphate appeared to be sphinganine, indicating the action of a hitherto unknown phosphatase. The label initially present in sphinganine was recovered at later time points in ceramide, which in its turn was gradually converted into sphingomyelin or cerebroside. When comparing O-acylation versus N-acylation in fibroblast cultures, using [9,10-³H]palmitic acid, the incorporation of label in sphingomyelin and ceramides was respectively approx. 20- and 100-fold lower than that in glycerolipids. Therefore, in our experiments labelling of sphingolipids due to N-acylation will represent approx. 5–1% of the radioactivity measured in these sphingolipids. Whereas the levels of free sphinganine remained almost unchanged, the label associated with sphingomyelin and cerebroside tended to accumulate with time (Figure 3).

The rates of dephosphorylation might be underestimated if desaturation of sphinganine to sphingene, which results in the loss of half of the radioactivity and in the formation of tritiated water, is extensive. Desaturation is nowadays believed to occur at the level of ceramide [22,23]. Given the continuous rise in labelling of sphingomyelin and cerebroside and the low amounts of labelled water-soluble products, the desaturation of N-acylated sphinganine does not seem to be high enough to cause a serious underestimation of the dephosphorylation rates.

Although we cannot exclude the possibility that some of the observed metabolites are derived from sphinganine possibly formed at the extracellular side of the plasma membrane or in the culture medium (cf. the presence of sphinganine in the culture medium after long-term incubation), several lines of evidence point to the uptake of the phosphorylated sphingoid base as such. First, at the early time points, the amount of label found in sphinganine 1-phosphate in the cell extracts reached that of the metabolites. Secondly, a considerable portion of the metabolites was formed by sphingosine-phosphate lyase, which has been localized to the outer aspect of the endoplasmic reticulum membrane [10]. Finally, the experiments performed by Spiegel and associates [4,6], using [³²P]sphinganine 1-phosphate, are not compatible with dephosphorylation followed by uptake and intracellular phosphorylation. Such a process would result in a dramatic decrease in the labelling of sphinganine 1-phosphate.

Once inside the cell, sphinganine 1-phosphate is rapidly and extensively remodelled via two pathways. The first pathway, catalysed by sphingosine-phosphate lyase, was already known and is located in the endoplasmic reticulum [3,15]. Such localization would be in agreement with a mere catabolic function. The other pathway, dephosphorylation, which has so far been reported only in tissue homogenates [3,24], appears to be physiologically relevant and not due to the action of some spurious phosphatase. Such a pathway was hitherto not con-

Figure 3 Cellular metabolism of the dephosphorylation products of sphinganine 1-phosphate

The radioactivity associated with cellular lipids (see Figure 1) that originate directly from the action of a sphingosine-phosphatase (sphinganine; ▲) or that are formed by further metabolism (□, ceramides; ▽, sphingomyelin; ○, sum of glycosphingolipids, mainly cerebrosides; ●, ceramide 1-phosphate; ■, sum of sphinganine and its further metabolites) is shown.

sidered and nothing is known with regard to the involved phosphatase(s) and their subcellular localization. Comparison of the rates of cleavage (Figure 2) with those of dephosphorylation (Figure 3) indicates that dephosphorylation is at least as active as cleavage, especially at the early time points.

The fast turnover of sphinganine 1-phosphate, as seen in this study, certainly favours a second-messenger role for this lipid intermediate. Such a role was somewhat in contradiction with the reported stability of sphinganine 1-phosphate in Swiss 3T3 cells [6] or B16 cells [9]. This stability is probably related to the position of the label. In the case of 3T3 cells, the label was present in the phosphate moiety. Hence, both metabolic pathways will generate a water-soluble radioactive breakdown product which will escape detection when analysing only lipid extracts. Therefore, if the rates of uptake and degradation are approx. equal, an apparent stability will be noted. The studies with B16 cells were performed with [^3H]sphinganine [9], which was phosphorylated rapidly. Cleavage of the sphinganine 1-phosphate formed results in labelled phosphoethanolamine, while dephosphorylation generates labelled sphinganine, which can be phosphorylated again. The concentration of detectable (i.e. labelled) sphinganine 1-phosphate would be steady when the rates of uptake and phosphorylation equal those of cleavage and dephosphorylation. Only at later time points, when the exogenous supply of labelled sphinganine decreased, were the levels of sphinganine 1-phosphate in B16 cells seen to drop. This was accompanied by the formation of labelled phosphatidylethanolamine [9] which indicates the action of sphingosine-phosphatase in these cells. In fact in our experiments, the amount of label present in cellular sphinganine 1-phosphate is relatively constant with time. Of course, differences in metabolism

in the various cells lines studied are likely to exist. Activities of sphingosine-phosphatase lyase [3] and sphingosine-phosphatase(s) (P. P. Van Veldhoven and G. P. Mannaerts, unpublished work), however, are of the same order of magnitude in most cultured cells or cell lines investigated, including fibroblasts, 3T3-L1, Swiss 3T3, Madin-Darby canine kidney, HeLa and cos-7 cells. Whether or not sphinganine 1-phosphate is somewhat less prone to degradation than its saturated analogue is not known. At least with regard to the cleavage reaction, no remarkable difference was noted [3].

Finally, we cannot exclude other possible metabolic conversions of sphinganine 1-phosphate, such as N-acylation to ceramide 1-phosphate or transfer of CDP-choline to yield sphinganyl phosphocholine. If occurring, they certainly represent minor pathways in the cell lines studied. At later time points in our experiments, a low degree of labelling of ceramide 1-phosphate was observed. Because of the delay in labelling, the radioactive ceramide phosphate was probably formed via phosphorylation of ceramide or via the action of a phospholipase D on sphingomyelin, and not via N-acylation of sphinganine 1-phosphate.

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