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Occurrence of ceramides and neutral glycolipids with unusual long-chain base composition in purified rat liver mitochondria

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Abstract The free ceramide content of rat liver mitochondria was found to be 1.7 nmol/mg protein and outer membranes contained a three-fold higher concentration than inner membranes. The mitochondrial content in neutral glycolipids was 0.6 nmol/mg protein. The long-chain bases found in free ceramides were d18:1 sphingosine, d18:0 3-ketosphinganine and t21:1 phytosphingosine in increasing order. In contrast, 3-ketosphinganine was the only base of glucosylceramide and lactosylceramide of inner membranes, whereas d18:1 sphingosine was the major long-chain base of glucosylceramide of outer membranes.

Key words: Mitochondrion; Ceramide; Glycosphingolipid; Ketosphinganine

1. Introduction

Ceramides have been recently identified as key signalling molecules which mediate many biological functions such as cell growth, differentiation, secretion and apoptosis [1]. Ceramide accumulation can be obtained in cells through a growing number of inducers such as tumour necrosis factor (TNF) α, dihydroxyvitamin D3, interferon γ, Fas ligands and irradiation [2]. Ceramide arising from de novo synthesis has also been clearly shown to be involved in the cellular response to inducers of apoptosis [3] and differentiation [4,5]. Exogenous ceramides have been demonstrated to exert a direct effect on isolated mitochondria by blocking the respiratory chain complex III [6] or by generating reactive oxygen species [7]. Although ceramide biosynthesis is known to be a stepwise process taking place mainly at the cytosolic face of the endoplasmic reticulum [8], direct acylation of long-chain bases has been suggested in liver mitochondria [9]. The report of sphingosine *N*-acyltransferase in mitochondria led us to investigate the presence of endogenous ceramides within mitochondrial membranes. As shown in the present study, ceramides and neutral glycosphingolipids can be clearly identified in rat liver mitochondrial membranes, using a recently developed method of analysis by gas chromatography–mass spectrometry (GC/MS) [10].

2. Materials and methods

2.1. Materials

Leupeptin, pepstatin, phenylmethylsulphonylfluoride (PMSF), bicinchoninic acid, ceramides containing non-hydroxy fatty acids (type III), ceramides containing hydroxy fatty acids (type IV), 2,3-dichloro-5,6-dicyanobenzoquinone, heptafluorobutyric anhydride, d18:1 erythrosphingosine, d18:0 dihydrosphingosine, t18:0 phytosphingosine and the salts for buffers were from Sigma, L'Isle d'Abeau, France. d18:0 3-Ketosphinganine and d20:0 sphingosine were obtained from Biovalley, Conches, France. LC-NH_2 cartridges were from Supelco, L'Isle d'Abeau, France. Analytical grade solvents and high performance thin layer chromatography (HPTLC) silica gel 60 plates were from Merck, Nogent, France. The capillary columns used for GC were purchased from Chrompack France, Les Ulis, France.

2.2. Methods

2.2.1. Isolation and purification of mitochondria. Livers freshly taken from rats were immersed in an ice-cold isolation medium made of 250 mM sucrose in 10 mM Tris–HCl, pH 7.4, containing 1 mM EDTA. Crude mitochondria were prepared by differential centrifugation as previously described [11]. For further purification, mitochondria were submitted to a mild ultrasonic treatment [12], layered on a 30% Percoll gradient and centrifuged [13]. Purified mitochondria were removed from the gradient and washed three times at $100\,000 \times g$ for 1 h in the presence of 1 μM leupeptin, 1 μM pepstatin and 0.1 mM PMSF.

2.2.2. Preparation of submitochondrial fractions. Outer and inner mitochondrial membranes were prepared from pure mitochondria as reported [11] and the resulting pellets were finally resuspended in 10 mM HEPES buffer, pH 7.4.

2.2.3. Marker enzyme assays. Monoamine oxidase was determined by the method of McCaman et al. [14]. Cytochrome *c* oxidase was assayed according to Rubin and Tzagoloff [15]. The activity of glucose-6-phosphatase was monitored by the method of Baginski et al. [16] and NADPH-cytochrome *c* reductase was measured according to Ernster et al. [17].

2.2.4. Protein determination. The protein concentration was determined in all fractions using bicinchoninic acid according to Smith et al. [18].

2.2.5. Extraction and purification of lipid fractions. Lipids were extracted from mitochondrial pellets and partitioned in chloroform–methanol–phosphate-buffered saline pH 7.4, 1:1:0.7 (by volume), as previously described [19]. The pooled ganglioside-containing upper phases were saved for further studies. The lower phases of partition were evaporated to dryness under reduced pressure, taken up in chloroform and the lipids were fractionated using solid-phase extraction on LC-NH_2 cartridges by a recently developed procedure [20]. The eluted fractions containing, respectively, free ceramides and neutral glycosphingolipids were evaporated under nitrogen. The recovery was monitored by TLC on HPTLC silica gel 60 plates along with known standards. Free ceramides were migrated, along with ceram-

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ides type III and ceramides type IV, in chloroform–methanol 50:3 (v/v), then the plate was visualised by charring for 10 min at 180°C after spraying with copper acetate 3% in phosphoric acid 8 M. The neutral glycosphingolipid fraction was spotted on a HPTLC plate, along with glucosylceramide and lactosylceramide purified from human melanoma tumours [21], then the plate was developed in chloroform–methanol–water 65:25:4 (by volume) and visualised with the orcinol–H_2SO_4 spray reagent. Sphingolipids were quantitated by assaying their constitutive long-chain bases with the fluorescamine method [22].

2.2.6. Synthesis of standard ceramides. Ceramides were synthesised, according to Anand et al. [23], by coupling oleic acid to standard long-chain bases d18:1 erythrosphingosine, d18:0 dihydrosphingosine, t18:0 phytosphingosine, d18:0 3-ketosphinganine and d20:0 sphingosine. Ceramides containing d18:1 3-ketosphingosine were prepared according to Iwamori et al. [24] by treatment with 2,3-dichloro-5,6-dicyanobenzoquinone of ceramides synthesised from pure sphingosine. These ceramides were then purified on LC-NH_2 cartridges as described [20] and analysed by GC/MS (see below).

2.2.7. Analysis of sphingolipids by GC/MS. The structure of the different samples was determined by GC/MS analysis of the heptafluorobutyrate derivatives of the products obtained after acid methanolysis [10]. Briefly, lipid samples were evaporated under nitrogen in heavy-walled Pyrex tubes (2.0 ml) with teflon-lined screw caps. After the addition of 0.5 ml of anhydrous HCl 0.5 M in methanol, the closed tubes were left for 20 h at 80°C. Then, the samples were evaporated to dryness under nitrogen, taken up in 200 μl acetonitrile and 25 μl of heptafluorobutyric acid, and derivatised for 15 min at 150°C. After cooling at room temperature, the samples were dried off under a stream of nitrogen, dissolved in acetonitrile and injected in the Ross injector of the GC/MS apparatus. The gas–liquid separation was carried out on a Carlo Erba GC 8000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB low bleed/MS capillary column, 0.25 μm film phase. The Ross injector was set at 280°C and the samples were analysed using a temperature programme from 90°C for 3 min, then 5°C/min up to 260°C followed by a plateau at this temperature. The column was coupled to a Riber 10-10H mass spectrometer (mass detection limit 2000 amu). Analyses were carried out either in the electron impact mode (ionisation energy 70 eV; source temperature 150°C) or in the chemical ionisation in the presence of ammonia (ionisation energy 150 eV; source temperature 100°C). Detection was performed for positive ions or for negative ions in separate experiments.

3. Results and discussion

3.1. Separation of sphingolipid classes

Following separation of lipid classes on LC-NH_2 columns as described [20], the ceramides and neutral glycosphingolipid fractions were applied to HPLTLC plates and visualised (see Figs. 1 and 2, respectively). As estimated by the assay of the constitutive long-chain bases using the fluorescamine method of Naoi et al. [22], the ceramide content of rat liver mitochondria was 1.66 ± 0.78 nmol/mg protein. The outer membranes that represent only one tenth of the mitochondrial proteins were found to be threefold enriched in ceramides (Table 1).

Fig. 2 shows the presence in rat liver mitochondria of neutral glycosphingolipids migrating as glucosylceramide and dihexosylceramide. Glucosylceramide was identified by its carbohydrate analysis and by its migration similar to that of standard glucosylceramide on borate-impregnated HPTLC plates [25] (not shown). Lactosylceramide was also identified

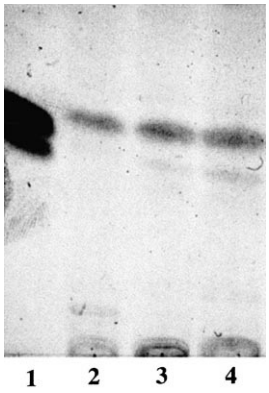

Fig. 1. Ceramides of rat liver mitochondria. Solvent: chloroform–methanol, 50:3, v/v. Visualisation: 3% copper acetate in 8% phosphoric acid, 5 min at 150°C. Lane 1, standard ceramides type III (non-hydroxy fatty acids); 2, outer membranes; 3, inner membranes; 4, whole mitochondria.

by its carbohydrate composition. The long-chain base assay of neutral glycosphingolipids showed a mitochondrial content at 0.62 ± 0.56 and 0.09 ± 0.12 nmol mg protein, respectively, for glucosylceramide and lactosylceramide, with an enrichment in the outer membrane as already found for ceramides (Table 1).

3.2. GC/MS analysis of sphingolipids

The analytical method used in our study to identify the components of sphingolipids involves first the derivatisation by heptafluorabutyric anhydride of all constituents released by acid methanolysis, then a single-step run of the whole sample on a gas chromatograph coupled to a mass spectrometer. The detection of ions specific for each class of compounds (i.e. fatty acids, long-chain bases, carbohydrates) allows the identification of each peak of the chromatogram.

Tables 2 and 3 give the fatty acid compositions of ceramides and neutral glycosphingolipids. The major fatty acids of ceramides were palmitic acid C16:0, oleic acid C18:1, stearic acid C18:0 and linoleic acid C18:2 in decreasing order. Long-chain species (> C20) were found in small amounts and only traces of α-hydroxy fatty acids were detectable. Except for a lower proportion of palmitic acid, the fatty acid composition was quite similar in glucosylceramides.

The analysis of mitochondrial sphingolipids showed that classical compounds of the sphingosine family were minor compounds relative to the fatty acid methyl esters. However, a correct molar ratio of long-chain bases to fatty acid methyl esters was possible only by accounting as a long-chain base a major peak with a retention time 2.5 min higher than that of linoleic acid methyl ester. A careful analysis of the fragmentation pattern (Fig. 3) showed ions at *m/z* 691 and 477 (difference of one heptafluorobutyric acid) together with ions at *m/z* 69, 119 and 169, characteristic of heptafluorobutyrate derivatives. In order to further characterise this compound, the sample was analysed using the chemical ionisation positive

Table 1
Content in ceramides and neutral glycosphingolipids of rat liver mitochondria

Sphingolipids	Outer membranes	Whole mitochondria	Inner membranes
Ceramides	2.83 ± 1.12	1.66 ± 0.78	1.45 ± 0.91
Glucosylceramide	1.56 ± 1.10	0.62 ± 0.56	0.60 ± 0.47
Lactosylceramide	0.21 ± 0.16	0.09 ± 0.12	0.08 ± 0.13

Results are expressed as nmol/mg protein. Data are given as mean ± S.D. of five different experiments.

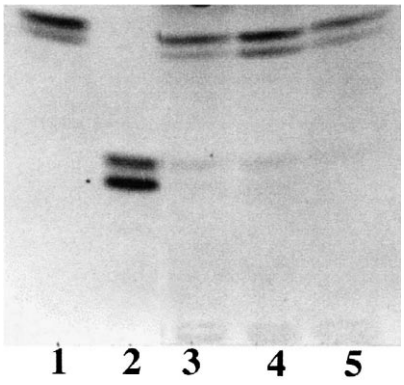

Fig. 2. Neutral glycosphingolipids of rat liver mitochondria. Solvent: chloroform–methanol–water, 65:25:4, by volume. Visualisation: orcinol–H_2SO_4 1 min at 150°C. Lane 1, glucosylceramide from human melanoma tumours; 2, lactosylceramide from human melanoma tumours; 3, outer membranes; 4, inner membranes; 5, whole mitochondria.

mode in the presence of ammonia and a $[M+NH_4]^+$ ion at *m/z* 740 (not shown) gave a mass of 722 for this major long-chain base. Based on the fragmentation pattern, the compound was thus identified as d18:0 3-ketosphinganine, and it was found as the major long-chain base in glucosylceramide of mitochondria (Table 3). Treatment with sodium borohydride of the glucosylceramide sample dissolved in dioxane prior to GC/MS analysis of the long-chain bases resulted in the disappearance of the peak identified as 3-ketosphinganine on the chromatogram along with a simultaneous increase of the d18:0 sphinganine peak. However, since the borohydride treatment can also give d18:0 sphinganine by reduction of 3-ketosphingosine, the fragmentation pattern of synthetic 3-ketosphingosine-containing ceramide [24] was determined and, after methanolysis and derivatisation with heptafluorobutyric anhydride, the results with major ions at *m/z* 463 and 493 ruled out the presence of 3-ketosphingosine. Moreover, the analysis of synthetic ceramides made by coupling standard 3-ketosphinganine with palmitic acid [23] gave a fragmentation pattern of the long-chain base similar to that of glucosylceramide purified from rat liver mitochondria. Thus, these data confirm the identification as d18:0 3-ketosphinganine of the major long-chain base of glucosylceramide purified from rat liver mitochondria.

By running heptafluorobutyrate derivatives of standard sphingosine-rich ceramides, a complete recovery of sphingosine relative to fatty acid methyl esters was ascertained, indicating that the low amount of d18:1 sphingosine found in the mitochondrial sphingolipids did not result from a loss that could occur during the analytical procedure. Compounds of the sphingosine family were identified by their intense ions at *m/z* 238, 290 and 459 for d18:1 sphingosine. Those of the sphinganine family were at 238, 292 and the additional ions at *m/z* 506 and 720 showed the presence of d18:0 sphinganine only. Phytosphingosines were identified by intense ions at *m/z* 238, 252, 256 and 264 characteristic of this family of long-chain bases, and intense ions at *m/z* 463 and 775 allowed the identification of t21:1 phytosphingosine.

As can be seen in Table 4, d18:1 sphingosine was a minor compound of mitochondrial free ceramides, and the most abundant long-chain bases were 3-ketosphinganine and phytosphingosine. Neutral glycosphingolipids gave a different long-chain base composition, showing the absence of phytosphingosine, and 3-ketosphingosine was almost the only long-chain base, except for the outer mitochondrial membranes in which a large amount of d18:1 sphingosine was seen.

The presence in rat liver mitochondria of sphingolipids having such unusual long-chain bases raises questions about the possible site of biosynthesis of these sphingolipids and the pathways that yield compounds such as 3-ketosphinganine-containing sphingolipid species. Long-chain bases have been shown to be synthesised and further acylated in the endoplasmic reticulum [26]. Although the report of *N*-acyltransferase activity suggests that the bases can be acylated in mitochondria [9], such a possibility requires further investigation. *N*-Glycosylation has been shown in mitochondria [27] and

Table 2
Fatty acid composition of free ceramides of rat liver mitochondria

Fatty acid species	Ceramides of outer membranes	Ceramides of whole mitochondria	Ceramides of inner membranes
C12:0	3.2 ± 0.5	2.8 ± 0.6	1.2 ± 0.6
C14:0	4.2 ± 0.4	2.0 ± 0.6	4.4 ± 0.4
C15:0	1.2 ± 0.7	n.d.	1.5 ± 0.5
C16:0	25.0 ± 2.1	20.3 ± 1.8	22.9 ± 3.3
C16:1	1.8 ± 0.5	2.2 ± 0.6	3.0 ± 0.6
C17:0	0.7 ± 0.6	0.8 ± 0.7	0.7 ± 0.6
C17:1	0.5 ± 0.5	n.d.	n.d.
C18:0	17.6 ± 1.3	17.6 ± 2.3	15.5 ± 1.5
C18:1	28.1 ± 2.2	29.1 ± 3.2	29.9 ± 3.0
C18:2	10.7 ± 0.9	9.8 ± 0.7	13.2 ± 0.4
C20:0	1.4 ± 0.5	n.d.	2.3 ± 0.4
C20:1-OH	n.d.	2.7 ± 0.7	1.6 ± 0.7
C22:0	2.1 ± 0.6	n.d.	2.0 ± 0.7
C32:0	0.4 ± 0.3	1.3 ± 0.4	0.5 ± 0.4
C32:1	0.5 ± 0.3	n.d.	n.d.
C24:0	1.1 ± 0.3	5.9 ± 0.4	0.7 ± 0.3
C24:1	1.2 ± 0.4	5.0 ± 0.3	n.d.
C24:1-OH	0.4 ± 0.3	n.d.	0.3 ± 0.3
C25:0	n.d.	0.3 ± 0.3	0.2 ± 0.2
C25:1	n.d.	0.6 ± 0.2	n.d.
C26:0	n.d.	0.1 ± 0.1	0.3 ± 0.1

Results are expressed as percentages of the total fatty acids for each sample. Data are given as mean ± S.D. of three different experiments (n.d., not detected).

Table 3
Fatty acid composition of neutral glycosphingolipids of rat liver mitochondria

Fatty acid species	Glc-Cer of whole mitochondria	Glc-Cer of outer membranes	Glc-Cer of inner membranes	Lac-Cer of inner membranes
C12:0	1.0 ± 0.3	1.4 ± 0.4	1.5 ± 0.3	0.3 ± 0.1
C13:0	1.0 ± 0.4	n.d.	n.d.	n.d.
C14:0	2.0 ± 0.3	1.0 ± 0.4	2.0 ± 0.2	1.6 ± 0.5
C15:0	1.5 ± 0.5	0.8 ± 0.6	1.0 ± 0.4	0.6 ± 0.6
C16:0	16.0 ± 2.2	22.5 ± 2.1	15.3 ± 1.3	19.2 ± 1.8
C16:1	2.5 ± 0.5	1.4 ± 0.5	1.0 ± 0.5	1.6 ± 0.4
C17:0	n.d.	n.d.	0.7 ± 0.5	n.d.
C18:0	9.0 ± 0.4	35.4 ± 0.2	9.2 ± 0.3	9.6 ± 0.3
C18:1	35.5 ± 4.1	21.3 ± 1.7	30.5 ± 2.7	49.4 ± 5.3
C18:2	21.5 ± 2.2	6.8 ± 0.5	26.8 ± 2.1	12.2 ± 1.3
C20:0	0.2 ± 0.2	1.5 ± 0.4	n.d.	n.d.
C21:0	n.d.	n.d.	1.5	n.d.
C22:0	3.0 ± 0.4	n.d.	n.d.	1.3 ± 0.5
C22:1	3.5 ± 0.4	2.7 ± 0.5	4.1 ± 0.3	2.9 ± 0.4
C32:0	1.0 ± 0.4	0.7 ± 0.5	1.2 ± 0.3	n.d.
C23:1	n.d.	n.d.	0.7 ± 05	n.d.
C24:0	2.5 ± 0.3	2.0 ± 0.4	3.6 ± 0.2	0.6 ± 0.4
C24:1	n.d.	n.d.	0.7 ± 0.3	0.6 ± 0.5
C25:0	0.7 ± 0.3	n.d.	n.d.	n.d.
C26:0-OH	n.d.	0.3 ± 0.3	n.d.	n.d.

Results are expressed as percentages of the total fatty acids for each sample. Data are given as mean ± S.D. of three different experiments (n.d., not detected).

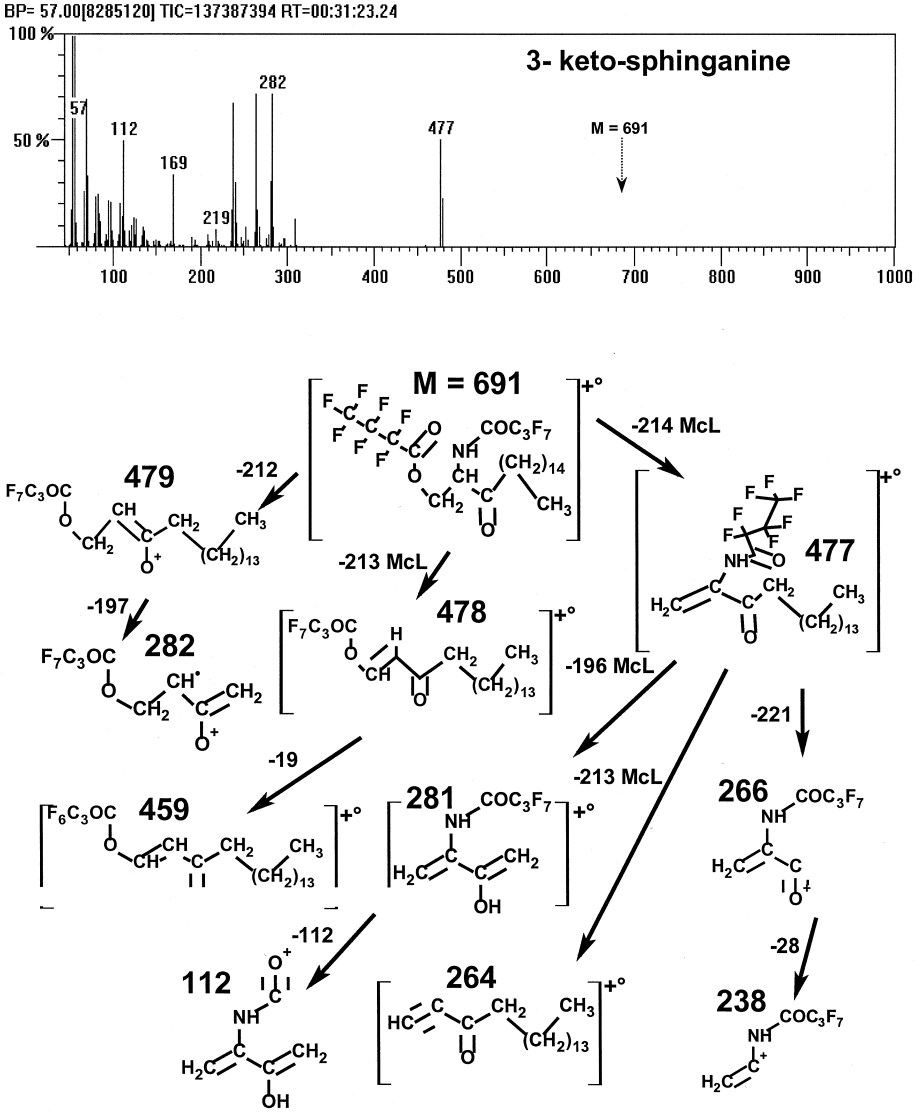

Fig. 3. Mass spectrum and fragmentation pattern of 3-ketosphinganine of rat liver mitochondria.

Table 4
Long-chain base composition of free ceramides and neutral glycosphingolipids of rat liver mitochondria

Long-chain base species	Free ceramides of whole mitochondria	Free ceramides of outer membranes	Free ceramides of inner membranes	Glc-Cer of whole mitochondria	Glc-Cer of outer membranes	Glc-Cer of inner membranes	Lac-Cer of inner membranes
d18:1 Sphingosine	18.4 ± 2.2	8.0 ± 0.5	4.2 ± 0.6	2.7 ± 0.5	51.0 ± 3.1	trace	trace
d18:0 Sphinganine	n.d.	n.d.	n.d.	n.d.	n.d.	trace	n.d.
d18:0 3-Ketosphinganine	28.8 ± 3.0	15.6 ± 2.3	41.7 ± 5.2	94.5 ± 10.2	26.0 ± 3.1	100.0 ± 1.2	100.0 ± 0.8
t21:1 Phytosphingosine	52.8 ± 4.6	76.4 ± 9.1	54.1 ± 4.3	n.d.	n.d.	n.d.	n.d.
Unidentified	n.d.	n.d.	n.d.	2.7 ± 0.3	23.0 ± 1.8	n.d.	n.d.

Results are expressed for each compound as percentages of total long-chain bases of the analysed sample. Data are given as mean ± S.D. of three different experiments (n.d., not detected).

we currently investigate the hypothesis of mitochondrial glycosyltransferases specific for glycosphingolipids, but the glycosphingolipids found in mitochondria can also have been glycosylated in other subcellular compartments before being transported to mitochondria. It is also possible that the mitochondrial ceramides derived from glycosphingolipids imported from other compartments and cleaved in the mitochondria by an enzyme such as the ceramidase recently reported by El Bawab et al. [28].

According to the currently known pathway of biosynthesis of long-chain bases in mammals, 3-ketosphinganine is made by condensation of serine and acyl CoA, then reduced to sphinganine with a high turnover and thus the keto form can be detected only in trace amounts [29]. Moreover, direct acylation of 3-ketosphinganine has never been reported. In order to determine whether this long-chain base pattern is specific for mitochondria, we are currently analyzing the sphingolipids in other purified subcellular fractions of rat liver to identify the constitutive long-chain bases.

Mitochondria are believed to be the targets of ceramide-mediated apoptosis [1]. It has been shown that ceramides containing sphingosine, are much more potent inducers of apoptosis than those containing sphinganine [7] or phytosphingosine [2]. Therefore, it would be unlikely that the mitochondria store a large amount of such apoptogenic ceramides, and this is consistent with the presence of sphingosine as a minor long-chain base of sphingolipids in rat liver mitochondria.

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