Natural ceramide is unable to escape the lysosome, in contrast to a fluorescent analogue

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Abstract Since the generation upon cell stimulation of the second messenger ceramide has been reported to occur in an endosomal/lysosomal compartment, we investigated whether ceramide formed in the lysosomes can escape this compartment. The metabolic fate of radiolabelled ceramide produced by intralysosomal hydrolysis of LDL-associated [*ceramide*-^{3}H]sphingomyelin or [*stearoyl-1*-^{14}C]sulfatide was examined in fibroblasts from control individuals and a patient with inborn lysosomal ceramidase deficiency (Farber disease). The behavior of this radioactive ceramide was compared to that of a fluorescent (lissamine-rhodaminyl) ceramide analogue deriving from sulfatide degradation. While in Farber cells the natural, radiolabelled ceramide remained completely undegraded and accumulated in the lysosomes, the fluorescent derivative was rapidly converted to sphingomyelin. These findings strongly suggest that, in contrast to fluorescent derivatives, endogenous long-chain ceramide is unable to exit from lysosomes, therefore making the lysosomal ceramide unlikely to be a biomodulatory molecule.

Key words: Ceramide; Lysosome; Farber disease; Sphingomyelin; Sulfatide

1. Introduction

Ceramide (*N*-acyl-sphingosine) is the backbone of all sphingolipids, which are essential constituents of eukaryotic membranes [1,2]. Ceramide is a precursor in the biosynthesis of all complex sphingolipids, as well as a product of their degradation. Recently, this lipid has emerged as an important intracellular second messenger, playing a key role in the signal transduction pathways of an increasing number of extracellular agents [3–6]. So far, sphingomyelin (SM, ceramide phosphocholine), a major structural component of the mammalian cell plasma membrane [7], has been proposed as the only sphingolipid source of the signalling ceramide.

While the biological effects of ceramide have been well characterized and some of its intracellular targets identified [3–6], the subcellular site of the generation of this lipid second messenger still remains controversial. In particular, it has been proposed that various biological responses are mediated by the formation of ceramide in acidic, endosomal/lysosomal compartments [8,9]. This model implies that the ceramide generated at this site is able to escape these compartments in order to physically interact with its molecular targets (which are not known to display an endosomal/lysosomal location). Consistent with this view, fluorescent short-chain analogues of ceramide seem to get across the lysosomal membrane [10–12]. However, the question whether natural, long-chain ceramide undergoes the same metabolic routing had not been investigated.

In the present study, we examined whether the ceramide formed in the lysosomes of cells having either a normal or a totally defective activity of lysosomal ceramidase (i.e. cells derived from Farber disease [13]) can get across the lysosomal membrane. Evidence is provided that, in contrast to ceramide derivatives labelled with a highly polar fluorescent probe, natural ceramide is unable to escape the lysosomal compartment. The implications of these findings with regard to the putative role of lysosomal ceramide in signal transduction phenomena are discussed.

2. Materials and methods

2.1. Chemicals

[*ceramide*-^{3}H]Sphingomyelin ([*ceramide*-^{3}H]SM, 400 mCi/mmol), prepared by catalytic tritiation of bovine brain SM, was obtained from CEA (Gif-sur-Yvette, France) [14]. Radiolabelled ceramide was obtained by hydrolysis of [*ceramide*-^{3}H]SM using *Bacillus cereus* sphingomyelinase (Sigma, Lisle d'Abeau, France) and was purified by TLC. [*stearoyl-1*-^{14}C]Sulfatide (55 mCi/mmol) was purchased from Aldrich (St. Louis, MO). *N*-Lissamine-rhodaminyl-(12-aminododecanoyl)-sphingolipids were synthesized as previously reported [15,16]. Silica gel 60 TLC plates (Art. 5721), TLC aluminium sheets (Art. 5553) and solvents were from Merck (Darmstadt, Germany). DMEM Glutamax, penicillin, streptomycin, and trypsin-EDTA were from Gibco BRL (Cergy-Pontoise, France); FCS was from Boehringer-Ingelheim (Gagny, France) and the serum substitute, Ultroser HY, from IBF (Villeneuve-la-Garenne, France).

2.2. Cell lines and cell culture

Human skin fibroblasts were derived from normal individuals, homozygous patients affected with late infantile metachromatic leukodystrophy (line Ale) or with Farber disease (line Moh). Fibroblasts of this Farber patient were previously shown to have a deficient (3.5%) acid ceramidase activity and to store large amounts of ceramide [17,18]. Alternatively, SV40-transformed fibroblasts from this patient

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Abbreviations: SM, sphingomyelin; LR12-CS, *N*-lissamine-rhodaminyl-(12-aminododecanoyl)-cerebroside 3-sulfate (or sulfatide); TLC, thin layer chromatography; FCS, fetal calf serum; LDL, low density lipoprotein

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and from control individuals were used [19]. Cells were grown as reported [19].

2.3. Preparation of LDL-associated [ceramide-^3H]SM and [^3H]ceramide

[*ceramide*-^{3}H]SM-labelled LDL and [^{3}H]ceramide-labelled LDL were prepared according to a previously reported procedure [14]. Alternatively, [^{3}H]ceramide-labelled LDL was prepared by incubating for 4 h at 37°C the LDL labelled with [*ceramide*-^{3}H]SM in the presence of *B. cereus* sphingomyelinase (1.5 U/mg LDL, in 5 mM $MgCl_2$, 0.15 M NaCl buffer, pH 7.4) to completely convert the SM to ceramide, and then by reisolating the LDL by ultracentrifugation.

2.4. Incubation of intact cells with sphingolipids

Before the experiments were initiated, the cells were grown for 24 h in DMEM medium containing 2% Ultroser HY, a serum substitute devoid of lipoproteins. Then, cells were incubated at 37°C for the indicated periods with medium containing 2% Ultroser and LDL-associated [*ceramide*-^{3}H]SM or [^{3}H]ceramide (the final concentration of LDL in the incubation medium averaged 50 μg apoB/ml, i.e. 500 000 dpm/ml) or LR12-CS (5 nmol/ml, introduced in ethanolic solution), or in the presence of 10% FCS containing [*stearoyl-1*-^{14}C]sulfatide (300 000 dpm/ml, also introduced in ethanolic solution). At the end of the 'pulse' period, cells were briefly washed at 37°C in medium containing 10% FCS. Fresh medium supplemented with 10% FCS was added to the cells and incubation continued for the indicated times. Then cells were washed thrice with phosphate buffered saline (PBS) containing BSA (2 mg/ml) and twice with PBS alone, and harvested using a rubber policeman. The cell pellets were stored at −20°C.

2.5. Subcellular fractionation studies

After a 24 h incubation with LDL-associated [*ceramide*-^{3}H]SM, followed by a 24 h chase, cells were washed with PBS, trypsinized, washed again and fractionated as described [20]. Postnuclear particles were resuspended in 0.6 ml of homogenization buffer adjusted with sucrose to a density of 1.3 g/ml, and layered under a continuous gradient of sucrose in homogenization buffer, prepared from two 5.5 ml solutions with densities of 1.1 and 1.3 g/ml. After centrifugation at 4°C and 50 000 × *g* for 18 h in a Kontron TST41.14 swinging rotor, 8–10 fractions were collected from the tube bottom, and the density was determined. The fractions were analyzed for radioactivity, protein content and marker enzymes: *N*-acetyl-β-hexosaminidase and succinate-tetrazolium reductase [14].

2.6. Lipid extraction and analyses

Cell pellets were homogenized in 0.6 ml distilled water and the lipids extracted as described [14,17,18]. Lipids were resolved by analytical TLC developed either in chloroform/methanol/water (100:42:6, v/v) up to 2/3 of the plate and then in chloroform/methanol/acetic acid (94:1:5, v/v) for radioactive lipids, or in chloroform/ethyl acetate/*n*-butanol/0.25% KCl/methanol (25:25:25:9:16, v/v) for LR12-lipids. The distribution of the radioactivity on the plate was analyzed using a Berthold LB 2832 radiochromatoscan. The various metabolic products were scraped and quantified by liquid scintillation or spectrofluorometry.

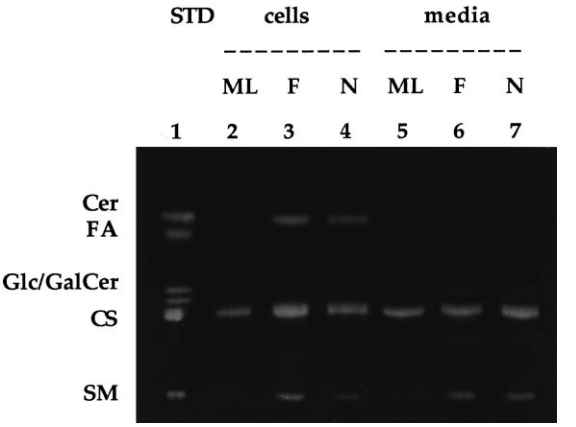

Fig. 1. Thin layer chromatogram of LR12-sphingolipids extracted from Farber disease fibroblasts incubated with LR12-ceramide. SV40-transformed fibroblasts from a control individual (N, lanes 4 and 7) and from a patient with Farber disease (F, lanes 3 and 6), and untransformed fibroblasts from a patient with metachromatic leukodystrophy (ML, lanes 2 and 5) were incubated for 72 h with 5 μM LR12-CS. Then, the culture medium was collected and the cells were washed and harvested. The lipids present both in the cells (lanes 2–4) and the culture media (lanes 5–7) were extracted and analyzed by TLC as described in Section 2. LR12-sphingolipid standards were simultaneously separated (STD, lane 1). The TLC plate was photographed under UV illumination. These data are representative of four independent experiments. SM, sphingomyelin; CS, sulfatide; Glc/GalCer, glucosyl/galactosylceramide; FA, fatty acid; Cer, ceramide.

3. Results

In previous studies [17], we identified a patient (Moh) affected with a very severe form of Farber disease. Cultured primary skin fibroblasts of this patient, who died at 3 days of age, exhibited a very high accumulation of ceramide, suggesting little (if any) residual acid ceramidase activity [17,18].

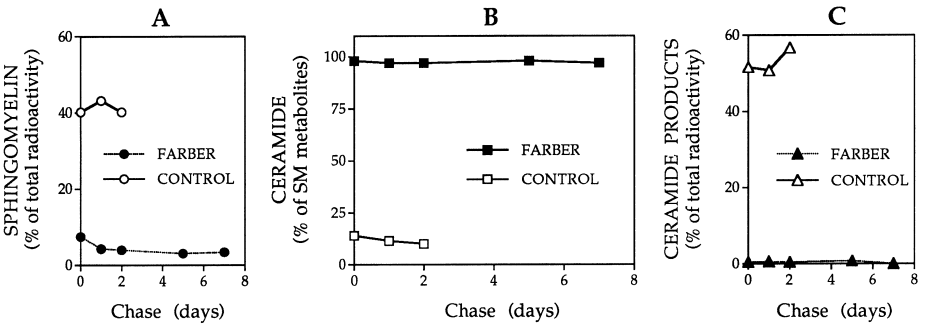

Fig. 2. Degradation of LDL-associated [*ceramide*-^{3}H]SM by normal but not Farber disease SV40-transformed fibroblasts. SV40-transformed fibroblasts from a control individual (open symbols) and from a patient with Farber disease (filled symbols) were incubated for 24 h with 0.6 μM LDL-associated [*ceramide*-^{3}H]SM. Then, at time zero the cells were washed and further incubated in medium devoid of radioactive lipid. At the indicated times, cells were washed and the lipids extracted and analyzed as described in Section 2. The amounts of total, including newly synthesized, SM (A) and ceramide products (C) [14,19] are expressed as percentage of the total cell-associated radioactivity, whereas the levels of intact [*ceramide*-^{3}H]SM (B) are expressed as percentage of the total lipid products of SM hydrolysis, i.e. by neglecting the SM itself but considering the ceramide as the substrate. At each time point, the data correspond to means ± S.D. of four separate experiments, except at time 7 days and for control cells (two separate experiments). The S.D. are too small to be seen.

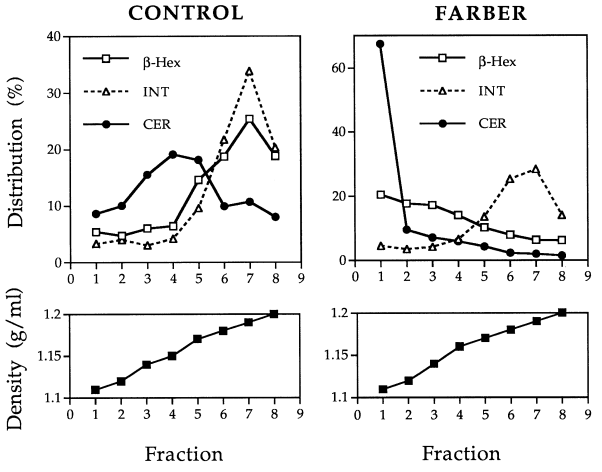

Fig. 3. Subcellular fractionation of fibroblasts from a control subject and a Farber disease patient incubated with LDL-associated [*ceramide*-^{3}H]SM. SV40-transformed fibroblasts derived from a control individual (left) and a patient with Farber disease (right) were pulsed for 24 h with LDL-associated [*ceramide*-^{3}H]SM, washed and further incubated for 24 h. Then, cells were homogenized, and postnuclear particles were prepared and layered under a continuous sucrose density gradient [20]. After centrifugation, eight fractions were collected and analyzed for density, radioactivity (CER), lysosomal (β-hexosaminidase; β-Hex) and mitochondrial (INT-succinate reductase) markers. The data correspond to a representative experiment; similar results were obtained in three other separate experiments.

This cell line was therefore selected for the experiments shown in the present study.

Initial experiments aimed at examining the metabolic fate of a polar fluorescent analogue of ceramide, LR12-ceramide in normal and Farber fibroblasts. In order to selectively target this lipid to the lysosomes, a metabolic precursor, LR12-sulfatide, was employed since previous observations had demonstrated that this sphingolipid is taken up by fibroblasts and hydrolyzed in the lysosomes [16,21]. As illustrated in Fig. 1 (lane 4), this fluorescent sulfatide was degraded by normal cells to galactosylceramide (faint band) and then to ceramide, which was converted to LR12-SM, presumably in the Golgi compartment [22–25]. This newly synthesized SM was released into the culture medium (lane 7). In contrast, in cells genetically deficient in the lysosomal cerebroside sulfatase activity, i.e. cells derived from a patient with metachromatic leukodystrophy, the LR12-sulfatide remained completely intact (Fig. 1, lanes 2 and 5), which corroborates the idea that the breakdown of this sphingolipid occurs only in the lysosomes. Of particular interest, fibroblasts from the severely affected Farber disease patient behaved exactly as control cells (lanes 3 and 6). Quantitative fluorometric determinations indicated that in control and Farber cells, the intracellular amounts of LR12-ceramide (35 vs. 38%) and LR12-SM (4% in both cases) were comparable. Also, Farber cells released into the medium similar amounts of LR12-SM as control cells. As previously observed [16,21], no free fluorescent fatty acid was detected.

Since the above observations on Farber fibroblasts, although consistent with data in the literature obtained with various fluorescent ceramides on other cell types [22,26,27], did not conform with the lysosomal storage of ceramide seen in this lipidosis [13,19], we next investigated the metabolic fate of naturally occurring, long-chain ceramides. Normal and Farber SV40-transformed fibroblasts were first incubated with LDL-associated [*ceramide*-^{3}H]SM in order to selectively target the sphingolipid to the lysosomes [14], then chased. Under these conditions, the ceramide released from radiolabelled SM was rapidly degraded in control cells (Fig. 2B), giving rise to various metabolic products (Fig. 2C) including newly synthesized SM (Fig. 2A) [14,17,19]. In contrast, in fibroblasts from a Farber disease patient, no significant degradation of [*ceramide*-^{3}H]SM could be observed, even after one week of chase (Fig. 2). In addition, the cell-associated radioactivity in these lipidotic cells remained constant over this chase period, strongly suggesting the absence of hydrolysis and efflux of undegraded ceramide. Very similar results were obtained when non-transformed cells were used (Table 1), or when cells were loaded with LDL-associated radiolabelled ceramide, i.e. either lipoproteins labelled with [*ceramide*-^{3}H]SM and then treated with bacterial sphingomyelinase or LDL directly labelled with [^{3}H]ceramide (Table 1). Moreover, Farber cells fed the natural, radiolabelled sulfatide ([*stearoyl-1*-^{14}C]sulfatide), the degradation of which is known to occur in the lysosomes [28,29], also accumulated the subsequently released ceramide (Table 1).

To examine the intracellular location of the radiolabelled, undegraded ceramide in Farber cells, subcellular fractionation experiments were performed after a chase period on cells loaded with LDL-associated [*ceramide*-^{3}H]SM. As shown in Fig. 3, a considerable part of the radioactivity present in control fibroblasts was distributed in fractions other than lysosomes because of extensive metabolism of SM and ceramide, and reincorporation in other organelles of intermediate equi-

Table 1
Fate of natural, radiolabelled ceramide from various sources in non-transformed and SV40-transformed fibroblasts from Farber disease

Cell line	Time of chase (days)	Non-transformed	SV40-transformed		
		[*cer*-^{3}H]SM	[*cer*-^{3}H]SM+SMase	[^{3}H]cer	[*stearoyl*-^{14}C]CS
Farber	0	99	87; 90	83 ± 2 (4)	89; 92
	1	98	88 ± 6 (3)	83 ± 2 (4)	ND
	2	99	90 ± 3 (3)	83 ± 5 (3)	87; 87
	4	ND	91 ± 3 (3)	89; 76	ND
Control	0	5.1*	20 ± 5 (3)	48 ± 5 (3)	19; 28

Non-transformed or SV40-transformed fibroblasts from control individuals and from a patient with Farber disease were incubated for 24 h with 0.6 μM LDL-associated [*ceramide*-^{3}H]SM ([*cer*-^{3}H]SM), *B. cereus* sphingomyelinase-treated LDL-associated [*ceramide*-^{3}H]SM ([*cer*-^{3}H]SM+SMase), LDL-associated [^{3}H]ceramide ([^{3}H]cer), or [*stearoyl*-^{14}C]sulfatide ([*stearoyl*-^{14}C]CS), washed and chased for the indicated intervals. Lipids were then extracted and analyzed as described in Section 2. The amounts of intact radioactive ceramide are expressed as percentages of either total cell-associated radioactivity (for cells loaded with [^{3}H]ceramide), or total lipid products (including fatty acid) of SM or galactosylceramide hydrolysis to consider the ceramide as the starting substrate.
ND, not determined. The asterisk refers to other published data [14,17].

librium density. In marked contrast, the radioactivity in Farber cells (which is mainly contributed by undegraded ceramide) clearly peaked in the lightest fraction, which was most coenriched in the lysosomal marker. There was also a considerable lightening of all lysosomes of Farber fibroblasts, as already reported [30]. This probably results from the storage of the hydrophobic lipid, ceramide. Taken together, the above data indicate that, independently of its origin, the natural, long-chain ceramide reaching or formed in the lysosomes of acid ceramidase-deficient cells accumulates there.

4. Discussion

Ceramide and some other sphingolipid derivatives are emerging as second messengers which play key signalling roles in a variety of cellular systems and exhibit pleiotropic biological responses [3–6]. While intracellular ceramide can originate from different sources (e.g. glycolipids or SM) and from a specific lipid (e.g. SM) through different enzymes exhibiting distinct subcellular localizations, the precise site of formation of the ceramide having signalling functions still remains a matter of debate. For instance, the signalling ceramide has been proposed to be generated from SM at the plasma membrane, in the inner leaflet [25,31] or in caveolae [32], and/or in the endosomal/lysosomal compartment [8,9]. Should the ceramide be formed in the lysosomes, this lipid must be assumed to be able to leave this compartment in order to exert its signalling functions. However, the question whether natural ceramide can indeed get across the lysosomal membrane has never been addressed. This issue is of crucial importance for a better understanding not only of the pathophysiology of inherited lipidoses such as Farber disease, but also of the mode of action of ceramide as an intracellular second messenger molecule.

Elegant studies by Pagano and coworkers using short-chain fluorescent (C6-NBD) derivatives of ceramide have suggested that ceramide could escape the lysosome and travel to the Golgi apparatus where it is metabolized to SM or glucosylceramide [12,22]. However, as pointed out by these authors, the mechanisms of metabolism and transport of these analogues may be unique to the short-chain sphingolipids because of their ability to undergo spontaneous monomeric transfer. The present and previous studies [16,21,27] indicate that the LR12-ceramide derivative exhibits a similar behavior, suggesting that in addition to the acyl chain length the presence of a polar fluorescent group can dramatically influence the trafficking route of sphingolipids. From the data obtained in this work and previous observations on either human fibroblasts [16,21] or rat oligodendrocytes [27] incubated either in the absence or presence of serum, the intracellular transport and metabolism of LR12-glycolipids can be interpreted as follows. After uptake, LR12-CS is internalized in the lysosomal compartment where it is degraded into ceramide. Evidence that hydrolysis of LR12-CS takes place solely in the lysosomes is provided by the complete inability of cerebroside sulfatase-deficient cells to metabolize this lipid. The subsequently formed LR12-ceramide, once transported to the Golgi compartment, is converted to SM, which reaches the plasma membrane by a vesicle-mediated process. At the cell surface, LR12-SM is released into the culture medium by back exchange, most probably because of the presence of the polar fluorophore. These metabolic routes were indistinguishable in normal and Farber disease fibroblasts, indicating that the trafficking of LR12-sphingolipids is not affected by the complete deficiency of lysosomal ceramide degradation.

The present study provides evidence that, in marked contrast to the behavior of the fluorescent ceramide derivative, natural radiolabelled ceramide is unable to leave the lysosome. This conclusion is supported by the following observations: (i) in normal fibroblasts, the long-chain ceramide delivered to, or formed within, lysosomes is rapidly degraded and its catabolic products are reutilized [14,17,29]; (ii) in severely deficient Farber cells, no degradation of lysosomal ceramide can be detected whatever its source (e.g. ceramide or SM delivered to the lysosomes through the apoB/E receptor-mediated endocytic pathway, or cerebroside sulfate); (iii) in Farber cells, natural ceramide accumulates in the lysosomal compartment. These findings lead to the conclusion that natural, long-chain ceramide cannot leave the lysosomes and that further catabolism to sphingosine and fatty acid, via the action of acid ceramidase, is needed to generate compounds able to escape these acidic compartments. This view is further strengthened by a recent report demonstrating accumulation in Farber fibroblasts of endogenously labelled ceramide [33].

The fact that natural ceramide is unable to escape the lysosomes has several implications. First, it demonstrates that at least in fibroblasts endogenous, long-chain ceramide undergoes different trafficking routes than fluorescent and/or short-chain ceramide derivatives. Therefore, although fluorescent analogues of sphingolipids have proven useful in delineating major pathways for sphingolipid synthesis, transport and secretion [11,12], data obtained with such derivatives should still be interpreted with caution, because they may not accurately reflect the fate of endogenous lipids. Second, differences in the severity of lysosomal ceramide storage observed among different Farber disease patients [17,19,33] are probably related to subtle differences in the residual effective acid ceramidase activity rather than defects or variations in the efflux of ceramide. Third, because of its inaccessibility to its cellular targets, ceramide produced in lysosomes is unlikely to play a signalling role. This would suggest that, if cell stimulation results in the activation of an acidic sphingomyelinase, the ceramide which is generated to act as a second messenger is probably not located in mature lysosomes but in another, as yet non-defined, subcellular compartment enriched in the above acidic sphingomyelinase.

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