Uptake and degradation of several pyrenesphingomyelins by skin fibroblasts from control subjects and patients with Niemann–Pick disease

Effect of the structure of the fluorescent fatty acyl residue

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Three fluorescent analogues of sphingomyelin (SPM), each containing pyrene in the fatty acyl residue, were synthesized and employed for the study of their mode of uptake by, and degradation within, intact cultured human skin fibroblasts. These were prepared by condensing sphingosylphosphocholine and the following fatty acids: pyrenedodecanoic acid (P12), pyrenesulphonylaminoundecanoic acid (PSA11) and pyrenepropenoic acid (P3:1). The cell association and catabolism of these SPM analogues by normal, Niemann–Pick-disease-Type-A and low-density-lipoprotein (LDL)-receptor-negative familial hypercholesterolaemia fibroblasts were investigated and compared with the metabolism of [cholinemethyl-¹⁴C]sphingomyelin. The catabolism of the fluorescent derivatives was monitored by measuring the appearance of the corresponding fluorescent ceramides. Two modes of uptake and degradation patterns were observed. Thus P12-SPM and radiolabelled SPM were taken up by LDL-receptor-mediated endocytosis when incubated with serum-containing medium, this conclusion being supported by the very low uptake by familial-hypercholesterolaemia fibroblasts, which lack the apolipoprotein-B/E receptor. After uptake, these compounds were metabolically degraded solely by the lysosomal sphingomyelinase, as evidenced by the fact that more than 98 % of the SPM remained undegraded in Niemann–Pick-disease cells. By contrast, PSA11- and P3:1-SPMs were taken up by a receptor-independent endocytic pathway, as indicated by the similar rates of uptake in control and familial-hypercholesterolaemia cells in the absence or presence of fetal-calf serum in the culture medium. The degradation of PSA11-SPM and P3:1-SPM was brought about, in the main, by the lysosomal sphingomyelinase, but also by a yet uncharacterized process. The latter catabolic pathway, active in Niemann–Pick-disease-Type-A fibroblasts, seems to differ from the neutral Mg²⁺-dependent sphingomyelinase whose activity was undetectable in homogenates of skin fibroblasts. The present study emphasizes the influence of the structure of the fatty acyl moiety of SPM on its association with lipoproteins and/or cell membranes and on its intracellular routing and metabolic degradation.

INTRODUCTION

Sphingomyelin (SPM; *N*-acylsphingosylphosphocholine) is a major lipid constituent of mammalian cell membranes, plasma lipoproteins, the myelin sheath and the erythrocyte stroma. SPM is actually a family of molecules differing by the structure of the long-chain sphingosine base and the fatty acyl residue [1]. Although the precise reactions involved in the biosynthesis of SPMs and their subcellular site are still not fully established [2], the hydrolysis of SPM is better known and is achieved by sphingomyelinase (sphingomyelin:choline phosphohydrolase, EC 3.1.4.12), which produces ceramide and phosphocholine [3]. Up until now two mammalian sphingomyelinases have been described: an acid lysosomal enzyme and a neutral membrane-bound enzyme, located mostly in neural tissues [3–5]. Niemann–Pick disease Type A is an inherited lysosomal disorder characterized by the accumulation of sphingomyelin in various organs as a consequence of almost complete deficiency of the lysosomal acid sphingomyelinase [2,3].

Cultured skin fibroblasts have been used to study the metabolism of SPM in intact living cells [6–14]. In these studies, SPM

was labelled either in the phosphocholine [6–9,11,12] or the ceramide moiety by a radiolabelled [8–10] or fluorescent fatty acid [10,13,14]. The SPMs were introduced into the cell-culture medium as liposomes or other undefined dispersions and were catabolized within the cells, although some metabolism was observed in lysosomal-sphingomyelinase-deficient (Niemann–Pick) fibroblasts [8,9]. Possibly owing to differences in the structures of the SPM analogues that were administered to the fibroblasts, quite conflicting conclusions were reported on the modes of entry of the respective compounds into the cells and on the nature of their catabolic pathways [6–14].

In previous investigations in our laboratories, a fluorescent pyrene derivative of SPM was synthesized, allowed to associate with the lipoproteins of fetal-calf serum and administered to fibroblasts, in which it was transported to the lysosomal compartment and subsequently degraded [13]. The present work was undertaken to investigate the influence of the structure of the fatty acid residue of SPM on its uptake and subsequent degradation by the cells. Several fluorescent derivatives of SPM were synthesized and administered to cultured skin fibroblasts from normal subjects and from patients with Niemann–Pick disease

Abbreviations used: SPM, sphingomyelin; P12-SPM, *N*-[12-(1-pyrene)dodecanoyl]sphingomyelin; PSA11-SPM, *N*-[11-(1-pyrene)sulphonylaminoundecanoyl]sphingomyelin; P3:1-SPM, *N*-[3-(1-pyrene)propenoyl]sphingomyelin; LDL, low-density lipoprotein.

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Type A. Their subsequent catabolism was studied and compared with the metabolism of a radioactive choline-labelled SPM. In addition, fibroblasts from a patient with apolipoprotein-B/E receptor-negative familial hypercholesterolaemia were also used in the present study to examine the involvement of the low-density-lipoprotein (LDL)-receptor-mediated pathway in the uptake and degradation of SPM.

MATERIALS AND METHODS

Chemicals

[*cholinemethyl*- ^{14}C]SPM (54 mCi/mmol) was purchased from New England Nuclear Corp. (Paris, France); 1-pyrene-dodecanoic acid (P12), 11-[(1-pyrenesulphonyl)amino]undecanoic acid (PSA11) and 1-pyrenepropenoic acid (P3:1) were from Molecular Probes (Eugene, OR, U.S.A.). Bovine brain SPM, *N*-hydroxysuccinimide and BSA were supplied from Sigma (St. Louis, MO, U.S.A.); *NN'*-dicyclohexylcarbodi-imide was from Aldrich (Steinheim, Germany); silica-gel 60 t.l.c. plates and aluminium sheets were from Merck (Darmstadt, Germany). All solvents and other reagents obtained from Merck or Prolabo (Paris, France) were of analytical grade.

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, trypsin/EDTA and fetal-calf serum were purchased from Seromed (Berlin, Germany). The serum substitute Ultrosor G was from IBF (Villeneuve-la-Garenne, France).

Cell culture

Cultured human skin fibroblasts from control individuals and from patients with Niemann-Pick disease Type A were obtained from our laboratory, from Dr. E. H. Schuchman and Dr. R. J. Desnick (Division of Medical Genetics, Mount Sinai Medical Center, New York, NY, U.S.A.) or Dr. G. Bach (Department of Human Genetics, Hadassah University Hospital, Jerusalem, Israel). Fibroblasts from a patient with LDL-receptor-negative familial hypercholesterolaemia were obtained from Dr. S. Eisenberg (Department of Medicine B, Hadassah University Hospital). Unless otherwise stated, cells were grown in a humidified air/CO₂ (19:1) atmosphere at 37 °C in RPMI 1640 medium containing L-glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (100 µg/ml) and heat-inactivated fetal-calf serum (10%). Cells were studied between passages 8 and 16.

Synthesis of fluorescent SPMs

The fluorescent derivatives of SPM (1-pyrene)propenoyl-SPM (P3:1-SPM), 12-(1-pyrene)dodecanoyl-SPM (P12-SPM) and 11-(1-pyrenesulphonyl)aminoundecanoyl-SPM (PSA11-SPM), were synthesized as previously reported [13,15,16], by condensing sphingosylphosphocholine (prepared from bovine brain SPM [15,17]) and the fluorescent fatty acid analogue. Alternatively the SPMs were synthesized as described by Ahmad *et al.* [18]. The fluorescent SPMs were purified by preparative t.l.c. and their concentrations were determined by phosphorus assay and/or measurement of their absorbance [13]. Also, the corresponding fluorescent ceramides were similarly synthesized from sphingosine [13]. Fig. 1 shows the chemical structure of the fluorescent SPMs.

Incubation of cultured cells with SPMs

For the metabolic studies, fibroblasts were trypsin-treated, plated into 60 mm-diameter Petri dishes and grown in a medium containing 10% (v/v) fetal-calf serum. Usually 2 days later the medium was removed and replaced by RPMI 1640 medium containing 2% Ultrosor G, a serum substitute. After 3 more

days, all the fibroblast cultures being confluent, the experiments were initiated.

A solution of the radioactive or fluorescent SPM in chloroform/methanol (2:1, v/v) was evaporated under N₂. The residue was dissolved in a small volume of 100% ethanol and mixed with sterile culture medium containing either 10% fetal-calf serum or only 2% Ultrosor G. These media were warmed at 37 °C for about 15 min and added to the fibroblast layers. The final concentration of fluorescent SPM in the incubation medium ranged from 3 to 6 nmol/ml and that of [*cholinemethyl*- ^{14}C]SPM was about 40000 d.p.m./ml, i.e. 330 pmol/ml (the concentration of the natural sphingomyelin contained in the lipoproteins of the fetal calf serum, when present, represented about 5–11 nmol/ml [9,19]). The final concentration of ethanol did not exceed 0.7% (v/v).

After incubating the fibroblasts for about 24 h at 37 °C, the experiments were terminated by removal of the culture medium and washing the cells three times with phosphate-buffered saline (PBS; 8 mM-sodium phosphate/0.14 M-NaCl, pH 7.4) supplemented with BSA (2 mg/ml), and then twice with PBS alone [20]. The fibroblasts were harvested by scraping with a rubber policeman and sedimented by low-speed centrifugation. The cell pellets were stored at –20 °C.

Lipid extraction and analyses

Cell pellets were suspended in 0.5 ml of distilled water and sonicated for 3 × 15 s (Soniprep MSE sonicator). An aliquot was taken for protein determination [21] and the remainder was extracted with 2.5 ml of chloroform/methanol (2:1, v/v) [22], vortex-mixed and centrifuged at 1000 g for 15 min.

When radiolabelled SPM was used, the upper aqueous phase of the Folch extract (containing the radioactive phosphocholine) was counted for radioactivity in a scintillation spectrometer (Packard Tri-Carb 4530) using Picofluor 15. An aliquot of the lower phase was also counted for radioactivity and the lipid extract was subjected to a mild alkaline hydrolysis [23] as follows: after evaporating the lower phase under a stream of N₂, the residue was dissolved in 0.25 ml of chloroform and 0.25 ml of methanolic 0.5 M-NaOH and incubated for 2 h at 37 °C. After adding 0.85 ml of chloroform, 0.25 ml of methanolic 0.5 M-HCl and 0.43 ml of water, mixing and centrifuging, the upper phase (containing the [^{14}C]choline label released from phosphatidylcholine) was counted for radioactivity. The organic phase of the Folch extract after mild alkaline hydrolysis (containing the sphingomyelin) was evaporated under N₂, dissolved in a reduced volume of chloroform/methanol and counted for radioactivity. Alternatively, for estimating the amounts of radioactive phosphatidylcholine and SPM in the extract, the lipids were resolved by analytical t.l.c. in chloroform/methanol/water (50:21:3, by vol.) and the plate analysed using a Berthold radiochromatocan densitometer.

When using the fluorescent derivatives of SPM, the upper phase of the Folch extract was discarded and the fluorescence intensity of the lipid phase was determined at the appropriate excitation and emission wavelengths (343 and 378 nm for P12-, 351 and 378 nm for PSA11- and 364 and 430 nm for P3:1-derivatives, respectively). This organic phase was then evaporated under N₂ and the residue dissolved in heptane/propan-2-ol/water for the separation of fluorescent catabolic SPM products [24,25]. The fluorescence intensity of the upper heptane-rich phase, containing fluorescent ceramide, was measured. For quantification, the fluorescence intensity of standards of the various fluorescent SPMs and ceramides in the appropriate solvents was recorded. Further identification of the fluorescent metabolites was performed by t.l.c. in chloroform/methanol/acetic acid (96:4:1, by vol.).

Fig. 1. Chemical structure of the fluorescent, pyrene-labelled SPMs

Table 1. Uptake of [*cholinemethyl*-¹⁴C]SPM and P12-SPM by cultured human skin fibroblasts

Normal, Niemann–Pick-disease-Type-A (NPD A) and LDL-receptor-negative familial-hypercholesterolaemia (FH) fibroblasts were incubated for 26–28 h with the radiolabelled or fluorescent SPM in the presence or absence of 10 % fetal-calf serum (FCS). The SPM concentration in the incubation medium approximated 40 000 d.p.m./ml (i.e. 330 pmol/ml) for the [*cholinemethyl*-¹⁴C]SPM and 4500 pmol/ml for the P12-SPM. The amounts of total cell-associated radioactivity or fluorescence were determined as detailed in the Materials and methods section. In the calculations, the amount of natural SPM contained in the fetal-calf serum (when present) was not considered. Note that, although the Table shows large differences between uptake values for the radioactively labelled versus the pyrene-labelled SPM, for both radioactive SPM and P12-SPM approx. 3–5 % and 1–1.5 % of the total SPM present in the incubation medium was taken up by the cells in the presence or absence of FCS respectively. For radiolabelled SPM the data are means $\pm$ S.D. for four and three to five separate experiments in the presence and in the absence of fetal-calf serum respectively. With P12-SPM, all the data correspond to means $\pm$ S.D. for three different experiments.

Sphingomyelin	FCS	Cell line...	Cellular uptake of sphingomyelin (pmol/mg of protein)			
			Normal 1	Normal 2	NPD A	FH
[<i>cholinemethyl</i> - ¹⁴ C]SPM	+		178 $\pm$ 49	180 $\pm$ 36	263 $\pm$ 94	14 $\pm$ 6
	–		67 $\pm$ 8	54 $\pm$ 16	85 $\pm$ 29	19 $\pm$ 6
P12-SPM	+		4500 $\pm$ 1400	3800 $\pm$ 1000	4100 $\pm$ 1900	170 $\pm$ 100
	–		1440 $\pm$ 600	1610 $\pm$ 700	1340 $\pm$ 600	130 $\pm$ 40

Table 2. Degradation of [*cholinemethyl*-¹⁴C]SPM and P12-SPM by cultured human skin fibroblasts

Normal, Niemann–Pick-disease-Type-A (NPD A) and LDL-receptor-negative familial hypercholesterolaemia (FH) fibroblasts were incubated with the radiolabelled or fluorescent SPM in the presence or absence of 10 % fetal-calf serum (FCS) as described in Table 1. The amounts of the catabolic products of [*cholinemethyl*-¹⁴C]SPM (i.e. [¹⁴C]phosphocholine and [¹⁴C]phosphatidylcholine) and P12-SPM (i.e. P12-ceramide) were determined as detailed in the Materials and methods section and are expressed as pmol/mg of cell protein. Alternatively they are presented as percentage of the total cell-associated radioactivity or fluorescence (in parentheses). Abbreviation: n.d., not detectable.

Labelled substrate	Degradation products	FCS	Cell line...	Amount of catabolic product (pmol/mg)			
				Normal 1	Normal 2	NPD A	FH
[<i>cholinemethyl</i> - ¹⁴ C]SPM	Phosphocholine	+		9.4 ± 5 (5.3)	7.0 ± 2.3 (3.9)	0.5 ± 0.4 (0.2)	0.3 ± 0.2 (2.1)
		–		2.0 ± 1 (3.0)	1.3 ± 0.4 (2.4)	0.3 ± 0.1 (0.4)	0.4 ± 0.2 (2.1)
	Phosphatidylcholine	+		118 ± 10 (66.2)	134 ± 10 (74.4)	2.6 ± 1.0 (1.0)	3.2 ± 1.0 (22.8)
		–		21 ± 3 (31.4)	21 ± 2 (38.9)	1.4 ± 0.4 (1.6)	4.4 ± 0.7 (23.1)
P12-SPM	P12-ceramide	+		1750 ± 560 (38.8)	1670 ± 290 (44.0)	80 ± 50 (1.9)	n.d.
		–		440 ± 200 (30.7)	610 ± 380 (37.9)	25 ± 10 (1.9)	n.d.

'In vitro' assays of sphingomyelinase activity

Sphingomyelinase activity in fibroblast homogenates was determined using PSA11-SPM as substrate in the presence of EDTA or MgCl₂ (5 mmol/l) as previously described [25,26].

RESULTS

SPM uptake and degradation

The uptake and catabolism of three fluorescent derivatives of SPM as well as the radiolabelled natural substrate were examined in human cultured skin fibroblasts from normal individuals and from Niemann–Pick-disease-Type-A and familial-hypercholesterolaemia patients. In order to investigate the potential role of lipoproteins in the delivery of SPM, the SPMs were administered to the cells either in the absence or in the presence

of fetal-calf serum. On the basis of cell-association and degradation data, two groups of SPM were indicated: one group includes the radioactive SPM and the P12 analogue (Tables 1 and 2); the other is represented by the PSA11 and P3:1 derivatives (Tables 3 and 4).

Concerning the first group (Table 1), the cell-associated radioactive SPM or P12-SPM in normal and Niemann–Pick-disease fibroblasts (no consistent differences were noted in the total SPM uptake between the two respective cells) was considerably greater than in familial-hypercholesterolaemia cells. Thus, in the presence of serum, the control and Niemann–Pick-disease fibroblasts incorporated 13–19 times more [*cholinemethyl*-¹⁴C]SPM and 22–26 times more P12-SPM than the corresponding receptor-negative fibroblasts (Table 1). Somewhat lower ratios were obtained in the absence of fetal-calf serum. When the serum was omitted from the incubation medium and replaced by Ultrosor G, a medium devoid of lipoproteins, the mean uptake of radiolabelled SPM or P12-SPM decreased by a factor of about 3 (Table 1). It is noteworthy that this was not true for familial-hypercholesterolaemia fibroblasts, in which the modification of the incubation medium was not accompanied by a significant change in the uptake rates (the ratio of uptake with or without fetal-calf serum was close to unity). A diminished uptake of [*cholinemethyl*-¹⁴C]SPM and P12-SPM in the latter fibroblasts (compared with controls) was also observed in the absence of Ultrosor G, i.e. in the presence of 10 % lipoprotein-deficient serum or even in RPMI alone (results not shown).

The degradation of the administered SPMs was followed by measuring the appearance of breakdown products in the cell extracts, i.e. radioactive phosphatidylcholine and water-soluble products (mainly phosphocholine) or fluorescent P12-ceramide (Table 2). As already reported [6–9,11,12], the major product of radiolabelled SPM hydrolysis after 24 h incubation was [¹⁴C]phosphatidylcholine, accounting for up to 74 % of the total cell-associated radioactivity in control cells. As clearly shown in Table 2, almost no metabolism of the introduced SPMs was detectable in Niemann–Pick-disease fibroblasts, whatever the catabolic product (water-soluble compounds, phosphatidylcholine, ceramide) examined, correlating with their deficient acid sphingomyelinase activity (see Fig. 3 below). In these mutant cells, the SPM that remained unhydrolysed after up to 30 h incubation represented 98–99 % of the total cell-associated

Table 3. Uptake of PSA11-SPM and P3:1-SPM by cultured human skin fibroblasts

Normal, Niemann–Pick-disease-Type-A (NPD A) and LDL-receptor-negative familial-hypercholesterolaemia (FH) fibroblasts were incubated for 23–24 h with the fluorescent SPM in the presence or in the absence of 10 % fetal-calf serum (FCS). The fluorescent SPM concentration in the incubation medium was 4000 and 3200 pmol/ml for the PSA11-SPM, and 6000 and 4000 pmol/ml for the P3:1-SPM, in the presence and in the absence of fetal-calf serum respectively. The amounts of cell-associated fluorescence were determined as described in the Materials and methods section. For results obtained in the presence of serum, the data are means ± S.D. for four and six separate experiments for PSA11-SPM and P3:1-SPM respectively. In the absence of fetal-calf serum the data correspond to a typical experiment.

SPM	FCS	Cell line...	Cellular uptake of sphingomyelin (pmol/mg of protein)		
			Normal	NPD A	FH
PSA11-SPM	+		1250 ± 600	1360 ± 500	920 ± 480
	–		1480	1950	1350
P3:1-SPM	+		6480 ± 2300	7300 ± 1500	5500 ± 2800
	–		6300	7800	4200

Table 4. Degradation of PSA11-SPM and P3:1-SPM by cultured skin fibroblasts

Normal, Niemann–Pick-disease-Type-A (NPD A) and LDL-receptor-negative familial-hypercholesterolaemia (FH) fibroblasts were incubated with the fluorescent SPMs in the presence or absence of 10 % fetal-calf serum (FCS) as described in Table 3. The amounts of the catabolic products, determined as detailed in the Materials and methods section, are expressed as pmol/mg of protein or, alternatively (in parentheses) as a percentage of the total cell-associated fluorescence.

Labelled substrate	Degradation products	FCS	Cell line...	Amount of metabolic product (pmol/mg)		
				Normal	NPD A	FH
PSA11-SPM	PSA11-ceramide	+		290 ± 90 (29.2)	90 ± 30 (6.6)	170 ± 80 (18.5)
		—		260 (17.6)	110 (5.6)	265 (19.6)
P3:1-SPM	P3:1-ceramide	+		1490 ± 590 (23.0)	270 ± 110 (3.7)	1050 ± 580 (19.1)
		—		1310 (20.8)	410 (5.2)	1100 (26.2)

Fig. 2. Thin-layer chromatogram of the lipids extracted from the cultured skin fibroblasts after incubation with P3:1-SPM

Normal, Niemann–Pick-disease-Type-A and familial-hypercholesterolaemia fibroblasts were incubated for 30 h with the fluorescent P3:1-SPM (6 nmol/ml) in the presence of 10 % fetal-calf serum. Cells were washed, scraped off and the lipids extracted as described in the Materials and methods section. In this particular experiment the protein amounts were 0.13, 0.31 and 0.24 mg, and the cell-associated fluorescence intensities corresponded to 0.77, 2.42 and 1.62 nmol in familial hypercholesterolaemia, Niemann–Pick-disease and normal fibroblasts respectively. The lipid extracts were evaporated under N_2 , dissolved in a small volume of chloroform/methanol and put on a silica thin-layer plate on an aluminium sheet. After development in chloroform/methanol/water (50:21:3, by vol.) up to the 2/3 of the plate, and then in chloroform/methanol/acetic acid (96:4:1, by vol.), the plate was photographed under Camag lamp u.v. irradiation (350 nm) using a Polaroid MP4 camera and a Polaroid 667 instant pack film. Lanes 1 and 7, marker of P3:1-SPM; lanes 2 and 6, marker of P3:1-ceramide; lane 3, familial hypercholesterolaemia; lane 4, Niemann–Pick disease Type A; lane 5, normal fibroblasts.

radioactivity or fluorescence. Similar data were observed in the absence of fetal-calf serum (Table 2). Although under these conditions somewhat less hydrolysis occurred in control fibroblasts, a marked metabolic deficiency was noted in Niemann–Pick-disease cells, the degradation rate in these cells representing only 5–6 % of the rates in controls.

Another set of data was obtained when using the PSA11-SPM and P3:1-SPM analogues (Tables 3 and 4). With these two derivatives, patterns of uptake and degradation different from

Fig. 3. Effect of pH on the 'in vitro' sphingomyelinase activity of control (□, △) and Niemann–Pick-disease-Type-A (NPD A; ■, ▲) cultured human skin fibroblasts

Sphingomyelinase activity was determined at different pH values using PSA11-SPM as substrate [25]. The assay mixture contained 100 nmol of fluorescent SPM/ml, 1 mg of Triton X-100/ml, 5 mmol of EDTA (□, ■) or $MgCl_2$ (△, ▲)/l, 250 mmol of sodium acetate buffer (pH 3.6–5.5) or Tris/maleate buffer (pH 5.2–8.5)/ml and enzyme (35–40 μ g of protein/ml) in a final volume of 0.2 ml. The enzyme activity was measured after 2 h incubation at 37 °C as described in the Materials and methods section. The results correspond to a typical experiment.

those using radioactive SPM or P12-SPM were observed. Thus there was no significant difference in the rates of uptake between the various cell lines, particularly between control and familial-hypercholesterolaemia fibroblasts (Table 3). Also, no difference was observed in the presence or absence of fetal-calf serum. In contrast with the previous group of SPMs, the uptake ratios with or without fetal-calf serum were close to unity. When monitoring the degradation of the latter two SPMs, the rates of hydrolysis in control cells, as measured by the appearance of fluorescent ceramide, were similar in both the presence and absence of serum (Table 4). However, these rates were slightly lower than those obtained with the radiolabelled SPM or P12-SPM. Furthermore, familial-hypercholesterolaemia cells did not show consistently different rates of degradation from the normal cells and, interestingly, some hydrolysis was noted in the Niemann–Pick-disease-Type-A fibroblasts. Although a catabolic defect was evident in the latter mutant cells (Table 4), the rates of degra-

dation reached up to 30 % of the controls. In order to avoid any misinterpretation of the data, the nature of the metabolic product in these cells was further carefully checked and identified as indeed being ceramide (Fig. 2).

'*In vitro*' sphingomyelinase activities

Since the degradation product of PSA11- and P3:1-SPMs in the experiments of Tables 3 and 4 was identified as ceramide, indicating the potential action of a sphingomyelinase, we searched for the presence of a non-lysosomal enzymic activity in fibroblast homogenates. As shown in Fig. 3, no sphingomyelinase activity other than the acid (pH 4.5–5.2), lysosomal one was detected at any pH, either in control or in Niemann–Pick-disease-Type-A fibroblasts, even in the presence of Mg^{2+} , i.e. under conditions optimal for the Mg^{2+} -stimulated brain enzyme [3–5,26].

DISCUSSION

The metabolism by human cultured skin fibroblasts of three fluorescent pyrene-labelled SPMs, structurally differing in the fatty acyl moiety, has been described and compared with that of [*cholinemethyl*- ^{14}C]SPM. The uptake and the subsequent degradation of the respective SPMs have been examined in normal, lysosomal sphingomyelinase-deficient (Niemann–Pick disease Type A) and apolipoprotein-B/E receptor-negative (familial hypercholesterolaemia) cells. The data clearly show that two groups of SPM analogues can be identified, depending on their mode of entry into the cells and on their respective metabolic pathways.

The P12 derivative of SPM exhibited a metabolic behaviour very similar to that of the radioactively labelled sphingolipid. Similarities were first observed in the mode of uptake: replacing the fetal-calf serum by a synthetic substitute resulted in a marked decrease in the rate of SPM uptake, as described by Kudoh *et al.* [9] and Vanier *et al.* [12] for radiolabelled SPM. Also, the catabolic pathway of P12-SPM seems to be the same as for [*cholinemethyl*- ^{14}C]SPM, i.e. the P12-SPM enters the lysosome, where it is hydrolysed by the acid sphingomyelinase in normal, but not in Niemann–Pick-disease-Type-A fibroblasts (less than 5 % hydrolysis in Niemann–Pick-disease cells as compared with controls). In this respect our observations agree well with previous reports using quite similar procedures on administration of the exogenous SPM [6,7,9,12]. When P12-SPM was used, less extensive hydrolysis than with the radiolabelled SPM was noted in normal cells. This result might be explained by a difference in the *in situ* substrate specificity of lysosomal sphingomyelinase, although *in vitro* the enzyme does not discriminate between the two substrates (T. Levade, S. Cherbu & S. Gatt, unpublished work). It is also possible that the P12-derivative might be sequestered in a non- (or poorly) metabolizable cellular compartment.

As previously observed using radioactive SPM [12], we showed that, when the fetal-calf serum in the incubation medium was replaced by Ultrosor G, the degradation rates of P12-SPM decreased, suggesting that the lipoprotein content of the medium influences not only the uptake, but also the metabolic utilization, of SPM [6,7]. The role of lipoproteins in the incubation medium is further indicated by the very low uptake of either [*cholinemethyl*- ^{14}C]SPM or P12-SPM by familial-hypercholesterolaemia fibroblasts. Since these cells lack the LDL receptor, this leads to the conclusion that, in the presence of fetal calf serum and in normal cells, the P12-SPM becomes associated with the apoprotein-B/E-containing lipoproteins and is taken up by the cells through the LDL receptor, targeting the internalized SPM to lysosomes [20]. However, an additional pathway, independent of lipoproteins, seems to take place for SPM delivery

to cells. This is supported by the following observations: in the presence of Ultrosor G, i.e. in the absence of lipoproteins, radioactive and P12-SPMs were taken up and degraded by normal fibroblasts, but at lower rates than when lipoproteins were present in the medium. Surprisingly, but as reported by Mazière *et al.* [11], this lipoprotein-independent mode of uptake was also defective in familial-hypercholesterolaemia fibroblasts.

A second metabolic group of SPMs is represented by the fluorescent analogues PSA11-SPM and P3:1-SPM (Tables 3 and 4). The mode of uptake of these derivatives is clearly independent of the LDL-receptor pathway of endocytosis, as demonstrated by the absence of any difference in the uptake rates between control and LDL-receptor negative fibroblasts. When cell-associated, these SPMs were actively hydrolysed, although the degradation rates were slightly lower than those observed with the first group of SPMs. Considerably less hydrolysis of PSA11-SPM and P3:1-SPM occurred in Niemann–Pick-disease than in control cells, indicating that lysosomal sphingomyelinase participated in this degradation. However, the Niemann–Pick-disease fibroblasts exhibited hydrolysis rates (up to 30 % of those in normal cells) much higher than those recorded with the first group of SPM (about 5 %). This suggests that the degradation of PSA11- and P3:1-SPMs seems to be achieved by two catabolic pathways, one lysosomal and another non-lysosomal. The lysosomal one, defective in Niemann–Pick-disease-Type-A cells, would account for about 70–80 % of the hydrolysis of these SPMs, the other one being responsible for the rest (20–30 %) of the production of fluorescent ceramide seen in Niemann–Pick-disease fibroblasts.

The nature of the non-lysosomal pathway is unclear. As the degradation product was ceramide, the possible presence of a neutral Mg^{2+} -stimulated sphingomyelinase activity in control and Niemann–Pick-disease fibroblast homogenates was examined *in vitro*; only the lysosomal enzyme activity was found. Since the PSA11-SPM used in these '*in vitro*' assays (which is the same as in Table 4) is a good substrate for the Mg^{2+} -dependent enzyme [25], we concluded that the fibroblasts do not have measurable neutral, Mg^{2+} -dependent sphingomyelinase activity, as suggested by Spence *et al.* [8]. Using liposomal SPM as substrate, Sutrina & Chen [10] reported the existence of a pH 6.5 sphingomyelinase in Niemann–Pick-disease fibroblasts. However, no indication was given about the EDTA sensitivity of this activity (which was about 10 times less than in controls). On the other hand, several reports have shown that, in fibroblasts: (i) there are two pH optima for the hydrolysis of SPM liposomes, the neutral activity not being inhibited by EDTA; and (ii) both acid and neutral Mg^{2+} -independent activities are deficient in Niemann–Pick-disease fibroblasts [27–29]. Therefore the hydrolysis of PSA11-SPM and P3:1-SPM is likely not catalysed by cellular neutral Mg^{2+} -dependent or -independent sphingomyelinases. An extracellular sphingomyelinase activity, present in fetal-calf serum [30], can be excluded since: (i) PSA11- and P3:1-SPMs were degraded in the absence of serum (Table 4), (ii) radiolabelled and P12-SPMs were not hydrolysed by Niemann–Pick-disease fibroblasts (Table 2) and (iii) no fluorescent ceramide was detected in the culture medium after incubation (results not shown). To try to account for the production of PSA11- or P3:1-ceramide by Niemann–Pick-disease cells, one could also speculate that transfer of phosphocholine from SPM to diacylglycerol [8,9] or CTP [31] occurred. If this is true, it would prove again that the metabolic pathways of the two groups of SPM herein described are different, since no evidence was provided for an active transfer reaction in Niemann–Pick-disease cells with the first SPM group (Table 2). Finally, the possibility of the combined action of a phospholipase D and a phosphatase cannot be ruled out.

The differences we observed in the modes of uptake and, perhaps consequently, of catabolism between the various SPM analogues might result from differences in their physicochemical properties. Thus, the R_f values of the fatty acids and ceramides labelled with the fluorescent probes were determined and showed that the P12 derivatives were less polar than the PSA11 or P3:1 analogues (minor differences were noted between the R_f values of the SPMs; results not shown). It is thus possible that modifications in the structure of the fatty acyl residue could affect the polarity of the ceramide moiety of SPM and alter the ability of these SPMs to bind to the lipoproteins or the cell surface. The influence of the structure of lipid derivatives on their transport across cell membranes, their cellular uptake and subsequent intracellular metabolism has already been documented for fluorescent fatty acids [32,33], fluorescent or radiolabelled ceramides [34,35] and fluorescent sulphatides [36]. Concerning SPM, Koval & Pagano [14] described the plasma-membrane labelling of fibroblasts using an analogue of SPM carrying a fluorescent probe (C6-NBD) different from pyrene. The metabolic fate of the SPM used by these authors is, however, quite difficult to compare with that of derivatives, since no mutant cells were examined in their study to characterize the degradation sites.

In conclusion, the above-presented data show the existence in fibroblasts of different pathways for the incorporation and metabolism of SPM. The use of different SPMs has provided evidence for the influence of the structure of the fatty acyl residue on the routing of SPM molecules. Although, under our experimental conditions, the degradation of P12-SPM is catalysed only by the lysosomal sphingomyelinase, the hydrolysis of two other pyrene-labelled SPMs is also achieved by an as yet uncharacterized process. Therefore, although all the fluorescent derivatives we have studied so far are suitable for diagnosing Niemann-Pick disease *in vitro*, we recommend the use only of P12-SPM as a substitute of the radioactive SPM both for estimating the lysosomal sphingomyelinase activity and diagnosing Niemann-Pick-disease-Type-A in intact living fibroblasts. As previously discussed [13], this fluorescent derivative might not be used for monitoring the further metabolic re-utilization of the liberated ceramide. However, it has the advantage over the radiolabelled analogue that it avoids the apparent SPM degradation process, also active in Niemann-Pick-disease cells and due to the phosphocholine transfer reaction, reported by other investigators [8,9]. Elsewhere, PSA11-SPM and P3:1-SPM may constitute useful tools for the study of the non-lysosomal metabolic pathway of SPM.

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