Predominance of the acylation route in the metabolic processing of exogenous sphingosine in neural and extraneural cells in culture

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The metabolic fate of exogenous [³H]sphingosine was investigated in five types of cultured cells: primary cultures of neurons and astrocytes, murine and human neuroblastoma cells and human skin fibroblasts. After administration of 40 nM [3-³H]sphingosine into a cell-conditioned medium containing fetal calf serum, all cell types rapidly and efficiently incorporated the long-chain base in a time-dependent fashion. In all cases, after a 120 min pulse, the amount of radioactivity taken up was in the range of the endogenous sphingosine content. However, unchanged [³H]-sphingosine represented only a very minor portion of the label incorporated into cells throughout the pulse period (10–120 min), indicating rapid and efficient sphingosine metabolism in these cells. Most of the [³H]sphingosine taken up was metabolically processed, either by degradation (assessed as ³H₂O release into

the culture medium) or by N-acylation (mainly to radioactive ceramide, sphingomyelin, neutral glycolipids and gangliosides). [³H]Sphingosine 1-phosphate accounted for less than 2% of the total radioactivity incorporated in all cases. Throughout the pulse period and in all cell types, ³H-labelled organic metabolites largely prevailed over ³H₂O, indicating that N-acylation is the major metabolic fate of sphingosine in these cells under apparently physiological conditions. These results are consistent with the notion that sphingosine has a rapid turnover in the cells studied, and indicate that regulation of the basal level of this bioactive molecule occurs mainly through N-acylation.

Key words: N-acylation, ceramide, sphingosine metabolism.

INTRODUCTION

Sphingosine [(2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol], the long-chain base present in cellular sphingolipids, is produced in cells by sphingolipid breakdown that leads first to ceramide and then to the free long-chain base and fatty acid (reviewed in [1,2]). Sphingosine that is formed can be either re-used for the biosynthesis of sphingolipids or degraded to ethanolamine phosphate and hexadecenal (reviewed in [3–5]) through a two-step process which involves phosphorylation at position 1 followed by lytic cleavage. In some cell types the conversion of sphingosine into *N,N*-dimethylsphingosine has also been documented [6,7].

In recent years, free sphingosine, as well as its metabolic derivatives ceramide, sphingosine 1-phosphate (sphingosine-1-P) and dimethylsphingosine, have emerged as potentially important bioregulators, able to modulate a variety of important cellular events (for reviews, see [8–11]). This has raised notable interest in the enzymic mechanisms that regulate the cellular content of these sphingoid molecules.

The recent evidence that the differentiation of neural cells involves ceramide and that sphingosine metabolism is implicated in the generation of bioactive ceramide [12–14] prompted us to investigate, in quantitative terms, the metabolic fate of free sphingosine in different cells of neural origin. The particular aim of this study was to evaluate which pathway (degradation or re-acylation) plays the predominant role in regulating sphingosine levels in neural cells under physiological conditions, i.e. in the presence of the presumably low sphingosine concentrations formed during sphingolipid breakdown. Thus sphingosine, at relatively low concentrations and isotopically radiolabelled at C-3, was administered to primary cultures of neurons and astrocytes, as well as to two lines of neuroblastoma cells (of murine

and human origin), and its metabolic processing was followed. In order to ascertain whether there is any specific neural cell pattern, the study was extended to human skin fibroblasts in culture.

EXPERIMENTAL

Chemicals

All reagents were of analytical grade, and solvents were redistilled before use. Culture media and fetal calf serum (FCS) were from Sigma (St. Louis, MO, U.S.A.); NaB³H₄ (6.5 Ci/mmol) was from Amersham International (Amersham, Bucks, U.K.); HPTLC silica gel plates were from Merck (Darmstadt, Germany); *Escherichia coli* *sn*-1,2-diacylglycerol kinase was from Calbiochem (La Jolla, CA, U.S.A.). D-Erythrosphingosine, isotopically tritiated at the C-3 position ([³H]sphingosine), was prepared and purified as described previously [15]. Its specific radioactivity was 1.88 Ci/mmol and its radiochemical purity, as assessed by HPTLC and autoradioscanning, was greater than 98%. Standard [³H]sphingolipids (ceramide, sphingomyelin, neutral glycolipids and gangliosides) were obtained as previously reported [15–17]. Standard dimethylsphingosine and sphingosine-1-P were generously provided by Professor Richard R. Schmidt (Faculty of Chemistry, University of Konstanz, Germany).

Cell cultures

Primary cultures of granule cells and astrocytes were prepared from the cerebellum of 8-day-old rats and cultured as previously

Abbreviations used: sphingosine-1-P, sphingosine 1-phosphate; FCS, fetal calf serum.

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described [18–20]. Both cell types were plated on poly(L-lysine)-coated dishes and cultured in supplemented basal modified Eagle's medium containing 10 % (v/v) FCS. Granule cells were used after 8 days in culture, when they were fully differentiated [18], and astrocytes after 10–12 days in culture, when type I cells prevailed and neurons were absent [20]. The murine neuroblastoma cell line, clone NB2a (Neuro2a; CCL-131; American Cell Type Culture Collection, Bethesda, MD, U.S.A.), and the cloned human neuroblastoma cell line SH-SY5Y (kindly provided by Dr. D. Fornasari, Center of Cellular and Molecular Pharmacology, C.N.R., Milan, Italy) were cultured in Dulbecco's modified Eagle's medium (Neuro2a) or RPMI 1640 (SH-SY5Y) supplemented with 10 % (v/v) FCS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml potassium penicillin G and 100 µg/ml streptomycin sulphate. Human skin fibroblasts, obtained from skin explants, were cultured in Eagle's minimum essential medium containing 10 % (v/v) FCS and antibiotics as described [21], and were used in a preconfluent state. All cells were grown in a humidified 5 % CO₂ incubator at 37 °C.

Administration of [³H]sphingosine to cultured cells

Studies were performed on cells (corresponding to 300 and 450 µg of protein/plate) plated on 60 mm dishes in a volume of 5 ml. [³H]Sphingosine solution was prepared in a cell-conditioned culture medium to avoid any possible transient stimulation of sphingolipid metabolism that would increase the cellular levels of sphingosine [22–24]. Immediately prior to the pulse, about half of the cell-conditioned medium was collected, centrifuged (1000 g, 15 min, 4 °C) and used to prepare the [³H]sphingosine solution. At the time of the experiments, the remaining medium was removed from the plates and 2 ml of conditioned medium containing 40 nM [³H]sphingosine (75 nCi/ml) was added to the dishes for times varying from 10 to 120 min. At the end of the pulse period, the medium was carefully collected and the cells were washed rapidly with cold PBS and harvested using a rubber scraper. The pulse medium was centrifuged as before and stored at –20 °C until processed for lipid extraction and fractional distillation (see below). Control experiments showed that, under these conditions, there was no appreciable change in the cellular levels of sphingosine in any of the cell types employed.

Lipid extraction and quantification

Total lipids were extracted from cells and culture media at 4 °C [17]. After partitioning, the organic phase was subjected to mild alkaline hydrolysis to remove glycerophospholipids. The aqueous and organic phases obtained were counted for radioactivity and analysed by HPTLC. The solvent systems used were: A, chloroform/methanol/water (55:20:3, by vol.); B, chloroform/methanol/32 % NH₄OH (40:10:1, by vol.); C, chloroform/methanol/0.2 % CaCl₂ (55:45:10, by vol.); D, chloroform/methanol/0.2 % CaCl₂ (50:42:11, by vol.); E, n-butanol/acetic acid/water (3:1:1, by vol.). After HPTLC, the plates were radioscanned with a Digital Autoradiograph. Since sphingosine-1-P partitions between the organic and aqueous phases, for quantification of [³H]sphingosine-1-P, aliquots of the total lipid extract were applied directly to silica-gel HPTLC plates and developed in solvent system E (see above). The recognition and identification of [³H]ceramide, [³H]sphingomyelin, ³H-labelled neutral glycolipids and [³H]gangliosides was performed as previously described [14,15]. [³H]Sphingosine-1-P was identified by co-migration with standard sphingosine-1-P in different chromatographic systems (solvent systems I–V in [25]).

Other methods

Radioactivity was determined by liquid scintillation counting or radiochromatoscanning (Digital Autoradiograph, Berthold, Germany) [16,17]. Ceramide and sphingosine contents were determined by the methods of Preiss et al. [26] and Ohta et al. [27] respectively. Sphingomyelin was determined, after perchloric acid digestion, as reported in [28,29]. Total protein was assayed [30] using BSA as the standard. ³H₂O, produced during [³H]-sphingosine degradation, was determined by fractional distillation of the culture medium under carefully controlled conditions, collecting the distilled fractions and measuring the radioactivity by liquid scintillation counting [31]. The volatile radioactivity of the culture medium was found to be present in the fraction distilled at 100 °C, moving to the aqueous phase after partitioning with chloroform.

RESULTS AND DISCUSSION

Sphingosine, produced in cells from sphingolipid degradation in the lysosomal compartment, leaves the lysosome and undergoes either phosphorylation by a sphingosine kinase linked to endoplasmic reticulum membranes [32] or N-acylation by ceramide synthase at the level of the external leaflet of the endoplasmic reticulum [33]. Therefore the cellular concentration of this molecule, which is particularly important for its bioregulatory role, depends on (a) formation from sphingolipid degradation, (b) phosphorylation and (c) N-acylation. Until now little has been known about the contribution of these routes to sphingosine levels in cells. In the present study we approached the problem by treating the cells with low concentrations (40 nM) of [³H]-sphingosine, thus mimicking the conditions of sphingosine release from lysosomes into the cytosol, and by following the metabolic fate of the long-chain base. Four types of cultured neural cells were employed, representative of differentiated neurons (cerebellar granule cells), proliferating glial cells (cerebellar astrocytes) and neural tumour cells (Neuro2a and SH-SY5Y cell lines), plus a non-neural cell type (human skin fibroblasts). The amounts of endogenous sphingosine, ceramide and sphingomyelin vary in these different cell types (Table 1). In primary cultures of differentiated granule cells, the levels of sphingosine and ceramide are 1.5–4.0-fold higher than in the other cells. Moreover, the content of sphingomyelin is markedly lower in the two tumour cell lines than in the other cell types. However, the amounts of these lipids are similar in astrocytes and fibroblasts.

In agreement with previous work showing sphingosine uptake by cells in culture [34,35], all cell types rapidly incorporated exogenously administered [³H]sphingosine, in a time-dependent

Table 1 Content of endogenous sphingosine, ceramide and sphingomyelin in the various cultured cell types used in this study

Data are given as pmol or nmol per mg of cell protein, and represent means ± S.D. of three independent experiments, performed in duplicate.

Cell type	Sphingosine (pmol/mg)	Ceramide (nmol/mg)	Sphingomyelin (nmol/mg)
Granule cells	180 ± 21	3.04 ± 0.28	12.82 ± 1.55
Astrocytes	124 ± 15	0.67 ± 0.08	13.31 ± 1.36
Neuro2a cells	106 ± 16	1.04 ± 0.11	3.65 ± 0.45
SH-SY5Y cells	95 ± 13	0.70 ± 0.09	1.43 ± 0.18
Fibroblasts	105 ± 15	0.95 ± 0.14	13.78 ± 1.89

Figure 1 Uptake of radioactivity by different cell types after administration of [³H]sphingosine for various times

[³H]Sphingosine (2 ml; 40 nM) was administered to cells plated on 60 mm dishes (corresponding to 300–450 µg of cell protein) in cell-conditioned medium containing 10% (v/v) FCS (see the Material and methods section). Total radioactivity is the sum of the radioactivity present in the organic and aqueous phases from the cell lipid extract plus the radioactivity present in water in the culture medium. Data are expressed as pmol/mg of protein, and are means of three independent experiments (performed in duplicate); S.D. values did not exceed 12% of the means.

fashion and with a similar pattern of uptake (Figure 1). After a 120 min pulse with [³H]sphingosine (40 nM; corresponding to 80 pmol per 300–450 µg of cell protein), the total amounts of sphingosine taken up were 114 ± 12 , 120 ± 10 , 78 ± 8 , 67 ± 8 and 112 ± 13 pmol/mg of cell protein in granule cells, astrocytes, Neuro2a cells, SH-SY5Y cells and fibroblasts respectively; these values correspond more or less to approx. 70% (in granule cells, Neuro2a cells and SH-SY5Y cells) and 100% (in astrocytes and fibroblasts) of the basal endogenous sphingosine content (Figure 1 and Table 1). However, in all cell types, cellular [³H]sphingosine did not represent the major portion of the label incorporated at any time investigated (Figure 1). In fact, even after a 10 min pulse, [³H]sphingosine accounted for less than 40% of the radioactivity present in all cell types; after a 120 min pulse, it represented only 1–6% of the total incorporated label. Thus, once incorporated into cells, [³H]sphingosine undergoes very rapid metabolic processing, and all the cell types were able to metabolize an amount of sphingosine approximately equivalent to the size of their sphingosine pool within 120 min of a pulse. This situation may mimic what happens in the cell with the sphingosine arising from sphingolipid degradation.

At all times investigated and in all cell types, most of the cell-associated radioactivity was recovered in the organic phase of the lipid extract, mainly as [³H]ceramide, [³H]sphingomyelin and neutral [³H]glycolipids; [³H]gangliosides were recovered in the aqueous phase in smaller amounts (Figure 2). Substantial amounts of [³H]sphingosine appeared to be incorporated rapidly

into ceramide. In fact, after a 10 min pulse, [³H]ceramide was the predominant organic metabolite in all cells, representing from 95% of the total ³H-labelled organic metabolites in SH-SY5Y cells to 62% in astrocytes. With increasing pulse times, the percentage of [³H]ceramide tended to decrease in favour of [³H]sphingomyelin, ³H-labelled neutral glycolipids and [³H]gangliosides. In all cell types, but especially in astrocytes and fibroblasts, [³H]sphingomyelin was the major complex sphingolipid produced from [³H]sphingosine throughout the pulse period. The distribution of radioactivity in the different complex lipids tended to reflect, especially for longer pulse times, the endogenous pattern of these compounds. In no case was the presence of [³H]dimethylsphingosine detectable.

Besides ³H-labelled molecules derived from sphingosine N-acylation, all cell types produced [³H]sphingosine-1-P and ³H₂O (Figure 3). In all cell types, [³H]sphingosine-1-P (Figure 3, left panel) was formed rapidly after [³H]sphingosine administration, being measurable throughout the pulse period; however, its content accounted for only a minor percentage (less than 2%) of the total incorporated radioactivity. ³H₂O, produced from the degradation of [³H]sphingosine, was the only ³H-labelled molecule (besides administered sphingosine) detectable in the pulse medium; it was already measurable after a 10 min pulse, and its concentration increased with time (Figure 3, right panel). The amounts of ³H₂O released into the medium from the five types of cultured cells were significantly different: astrocytes produced the greatest and neuroblastoma cells the smallest amounts.

Figure 2 Incorporation of radioactivity into ceramide and complex sphingolipids after exposure of different cell types to [3 H]sphingosine

[3 H]Sphingosine (2 ml; 40 nM) was administered to different cell types (300–450 μ g of protein) for different time periods. The data are means of three experiments (performed in duplicate); S.D. values did not exceed 15% of the means.

Figure 3 Incorporation of radioactivity into sphingosine-1-P (left) and water (right) in the five cell types after different pulse periods with [3 H]sphingosine

[3 H]Sphingosine (40 nM; 2 ml; added to 300–450 μ g of cell protein) was administered in FCS-containing medium for times varying from 10 to 120 min. The data are means of three experiments (performed in duplicate); S.D. values did not exceed 15% of the means. Abbreviation: SPH-1-P, sphingosine-1-P.

All cell types processed exogenous sphingosine efficiently via both N-acylation (measured as [3 H]sphingolipids) and complete degradation (mainly measured as 3 H $_2$ O). The results obtained demonstrate that, throughout the pulse period and in all cell types, the products of sphingosine N-acylation predominated over degradation metabolites (Figure 4). In fact, after a 120 min pulse, when the percentage degradation was greatest, only 28% of the incorporated [3 H]sphingosine in cerebellar astrocytes, 15% in cerebellar granule cells and fibroblasts and less than 8% in the neurotumour cells had undergone degradation.

The rapid incorporation of [3 H]sphingosine first into ceramide, and later into complex sphingolipids, is in agreement with previous evidence indicating that sphingosine produced from the hydrolysis of complex sphingolipid is mainly re-incorporated into sphingolipids [36,37], although with differing efficiencies in different cells [38]. All this evidence supports the notion that, under our experimental conditions, the metabolism of exogenous [3 H]sphingosine seems to mimic that of sphingosine normally produced during sphingolipid catabolism in the cell.

The results reported here, i.e. that N-acylation prevails in the metabolism of exogenous sphingosine in cells, differ from those in other studies that demonstrated a prevalence of sphingosine phosphorylation and degradation [24,39,40]. This cannot be explained in terms of cell specificity, as the five types of cells we used were extremely heterogeneous: they came from different species (mouse, rat, human), were of neural and extraneural origin, were proliferating, differentiated or tumour cells, and were characterized by different sphingolipid patterns. A reasonable explanation of this difference could lie in the amount of

Figure 4 Distribution of radioactivity between metabolites derived from degradation and N-acylation of [³H]sphingosine after pulses of 10 min, 1 h and 2 h

Each bar represents total radioactive metabolites, determined as the sum of N-acylation products (open bars) and degradation products (cross-hatched bars). Data are expressed as pmol/mg of protein.

exogenous sphingosine administered to the cells. In fact, in the investigations cited [24,39,40], micromolar concentrations of sphingosine were used, high enough to result in sphingosine incorporation in amounts corresponding to 10–100 times the cellular long-chain base content. Moreover, recent reports [41,42] have shown that, in various cell types, the ratio between N-acylation and degradation products after a 2 h pulse decreases with increasing concentrations of sphingosine (from 40 nM to 10 μ M). These data could suggest that, when the intracellular concentration of sphingosine greatly exceeds the physiological capacity of the cells to acylate it, more sphingosine is routed towards degradation.

In conclusion, the results presented here suggest that the intracellular sphingosine concentration is regulated mainly by N-acylation processes and by the overall capacity of the cell to biosynthesize sphingolipids. Hence this mechanism will play a critical role in the concurrent control of the intracellular concentrations of sphingosine and ceramide, and thus in the expression of their bioregulatory potential.

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