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Formation of bioactive sphingoid molecules from exogenous sphingomyelin in primary cultures of neurons and astrocytes

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Abstract Exogenous sphingomyelin, radiolabelled at the sphingosine moiety, was administered to primary cultures of cerebellar granule cells and astrocytes for different pulse times (20 min–2 h) and the fate of the radioactivity was followed. Ceramide was the main metabolic product in both cells, whereas sphingosine, glucosyl-ceramide and gangliosides GM3 and GD3 were produced only in astrocytes. When endocytosis was prevented and the lysosomal apparatus inactivated, ceramide formation was reduced slightly in granule cells and almost completely blocked in astrocytes, with disappearance of sphingosine, glucosyl-ceramide, GM3 and GD3. These data indicate that (a) ceramide is rapidly produced in cerebellar granule cells and astrocytes, presumably at the level of the plasma membrane in the first cell type, and of the lysosomes in the second one; (b) sphingosine is produced in cerebellar astrocytes by lysosomal sphingomyelin degradation and is partly reused for glucosyl-ceramide and ganglioside biosynthesis.

Key words: Sphingomyelin; Sphingomyelin metabolism; Lipid second messenger; Neural cells in culture

1. Introduction

Sphingomyelin, a major sphingolipid constituent of cell membranes in mammals, is preferentially concentrated in the outer leaflet of the plasma membrane [1]. In the last few years evidence emerged indicating the involvement of sphingomyelin degradation in producing sphingoid molecules (ceramide, ceramide-1-phosphate, sphingosine, sphingosine-1-phosphate, etc.) with potential second messenger function [2–7].

Sphingomyelin degradation is initiated by the action of sphingomyelinase (SM-ase), with formation of ceramide and phosphocholine [2,8]. Both neutral and acidic SM-ases occur in different tissues [9–12]. Neutral SM-ases were found in the plasma membrane and the cytosol. The plasma membrane associated enzyme is Mg^{2+}-dependent, particularly abundant in the nervous system and enriched in the grey matter [11–13]. In cultured neuroblastoma cells it was found to be extracellularly oriented [14]. The only known neutral cytosolic SM-ase, purified from HL-60 cells, is a Mg^{2+}-independent enzyme, activated by cell treatment with 1α,25-dihydroxyvitamin D_3 [15]. The acidic SM-ase appears to be located in the lysosomes [9,10]. Both neutral and acidic SM-ases were purified and characterized [16–18] from human and rodent brain. Notwithstanding these enzymological studies, little is known on sphingomyelin degradation in intact living cells of neural origin.

Along the research line aimed at ascertaining the possible involvement of sphingolipid-mediated signal transduction in neural differentiation, we studied the metabolism of sphingomyelin, with particular attention to the formation of ceramide and sphingosine, in primary cultures of cerebellar neurons and astrocytes. Both cell lines, obtainable at a high degree of homogeneity, are proper models to study differentiation processes in vitro. The cell cultures were exposed to exogenous sphingomyelin, radiolabeled at the level of the sphingosine moiety, and the formation of radioactive metabolites was followed. Conditions were used capable to distinguish between the lysosomal and extralysosomal pathway of sphingomyelin degradation. The rationale of this approach is based on the evidence [19–21] that exogenous sphingomyelin is taken up and metabolically processed by cultured cells, mimicking the behaviour of the endogenous compound.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade and solvents were redistilled before use. Basal modified Eagle's medium (BME) and FCS (heat inactivated before use) were from Flow Laboratories (Irvine, Scotland); bovine brain SM, poly-L-lysine, bovine serum albumin and phosphorus standard solution from Sigma (St. Louis, MO, USA); [^{3}H]$NaBH_4$ (6.5 Ci/mmol) from Amersham International (Amersham, Bucks, UK). SM was isotopically radiolabelled at the level of the sphingosine moiety ([Sph-^{3}H]SM), as previously described [22]. Its specific radioactivity was 0.35 Ci/mmol, and the radiochemical purity, assessed by HPTLC and autoradioscanning, better than 98%. Standard [^{3}H]Cer, [^{3}H]Sph, [^{3}H]Glc-Cer and [^{3}H]gangliosides (GM3, GM2, GM1, GD1a, GD1b, and GT1b) were obtained as previously reported [23–24].

2.2. Cell cultures

Primary cultures of granule cells and astrocytes were prepared from the cerebellum of 8-day-old rats and cultured as previously described [25–27]. Both cells were plated on 60-mm diameter poly-L-lysine coated dishes and cultured in BME containing 10% FCS. Granule cells were used at the 8th DIC, when they were fully differentiated [25], and astrocytes at the 10th–12th DIC, when type I cells prevailed and neurons were absent [27]. In the used cultures, both types of cells represented about 95% of the cell population. Unless otherwise stated, cells were grown in a humidified 5% CO_2 incubator at 37°C.

2.3. Treatment of cultured cells with [Sph-^{3}H]SM

Radioactive SM was dissolved in a small volume of absolute ethanol and mixed with sterile culture medium without FCS at the final concentration of 2×10^{-6} M (0.7 μCi/ml). At the time of experiment, dishes were washed three times with temperature conditioned BME without FCS and incubated for different periods of time (pulse) (up to 2 h) in the same medium (2 ml/dish) containing [Sph-^{3}H]SM. Treatment with

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Abbreviations: SM, sphingomyelin; Sph, sphingosine; Cer, ceramide; Glc-Cer, glucosylceramide; SM-ase, sphingomyelinase; DIC, day in culture; HPTLC, high-performance thin-layer chromatography; FCS, fetal calf serum. Gangliosides were named according to Svennerholm [34].

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[Sph-^{3}H]SM was performed in the absence of fetal calf serum in agreement with previous experience [23,24] and the recent report [28] indicating that under this condition taken up SM is able to undergo both the lysosomal and extralysosomal processing pathways. The culture medium was then removed and the cells washed, first with the above culture medium containing 10% FCS in order to remove loosely bound radioactivity, and then with phosphate-buffered saline (three times). Cells were then harvested by scraping, lyophilised and stored at −20°C. The process of endocytosis was blocked by performing treatment at 4°C and lysosomal activity inhibited by adding 100 μM chloroquine 1 h prior and during pulse [24].

2.4. Lipid extraction and purification

Total lipids were extracted and partitioned as previously described [24]. The obtained aqueous and organic (this one after a mild alkaline hydrolysis) phases were counted for radioactivity and analyzed for individual constituents (see below). The recognition and identification of the individual [^{3}H]metabolites (Cer, Glc-Cer, Sph and individual gangliosides) was performed as described [23,24].

2.5. Lipid separation by HPTLC

The different lipids present in the organic and aqueous phases were separated by HPTLC, using chloroform/methanol/water (55:20:3, by vol.) or chloroform/methanol/32% NH_4OH (40:10:1, by vol.), for the organic phase and chloroform/methanol/0.2% $CaCl_2$ (55:45:10, by vol.) for the aqueous phase. After HPTLC, the plates were radioscanned with a digital autoradiograph (Berthold, Germany).

2.6. Colorimetric methods

SM was determined, after perchloric acid digestion, according to the procedure of Bartlett [29], as modified by Dodge and Phillips [30]. Total proteins were assayed [31] using bovine serum albumin as the standard.

3. Results

As shown in Fig. 1, the uptake of [Sph-^{3}H]SM by cultured granule cells and astrocytes was time-dependent in the 20–60 min pulse period examined. Both cell types incorporated similar amounts of radioactivity, astrocytes showing a slightly higher uptake. At all investigated times and in both cell types, radiolabelled SM accounted for the large majority of incorporated radioactivity (>85%). After 60 min pulse the amount of bound [Sph-^{3}H]SM accounted for about 5.4 and 3.5% of the endogenous SM in granule cells and astrocytes, respectively.

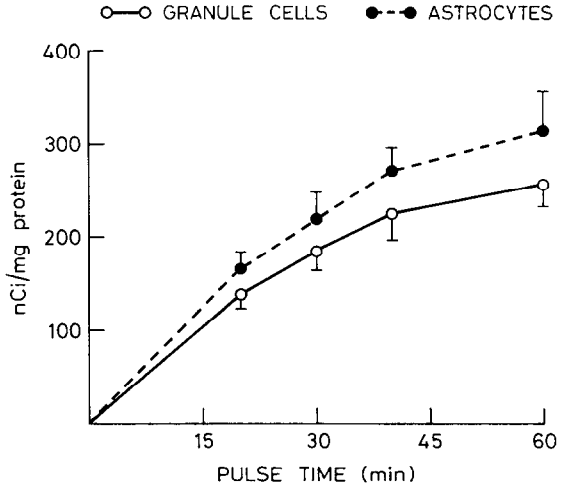

Fig. 1. Time course of stable radioactivity incorporation into cerebellar granule cells and astrocytes in culture, after exposure to 2×10^{-6} M [Sph-^{3}H]SM for different periods of time. The data presented are the mean ± S.D. of three experiments and are expressed as nCi/mg protein.

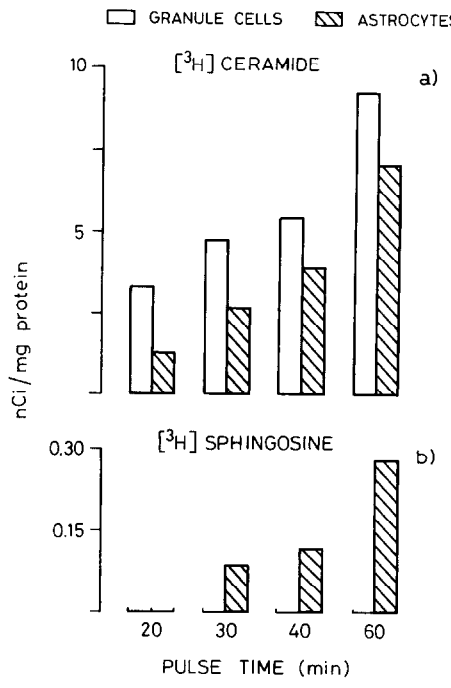

Fig. 2. Incorporation of radioactivity into Cer and Sph by cerebellar granule cells and astrocytes in culture after exposure to 2×10^{-6} M [Sph-^{3}H]SM for different pulse times. The data presented are the mean of three experiments and are expressed as nCi/mg protein. S.D. values never exceeded 12% of the mean values.

In both granule cells and astrocytes [^{3}H]Cer was the major metabolite from exogenous SM. As shown in Fig. 2a, it was produced early (already at 20 min) after exposure, in a time-dependent fashion, and in higher amounts by neurons than astrocytes. In cultured astrocytes [^{3}H]Sph started being measurable after a 30 min pulse (Fig. 2b), its content increasing with time but being always 25- to 30-fold lower than that of [^{3}H]Cer. Conversely, no [^{3}H]Sph could be detected in cultured granule cells up to 2 h. The subcellular site of formation of sphingoid metabolites from exogenous SM was inspected with experiments carried out at 4°C or in the presence of 100 μM chloroquine using a pulse of 2 h. The results, shown in Fig. 3, demonstrate that both conditions affected the formation of radiolabelled sphingoid molecules only partially in granule cells but very markedly in astrocytes. In particular, the radioactivity incorporated into Cer (as percent of the control cells) was 83% and 10% in the presence of chloroquine, and 60% and 1.5% at 4°C, in granule cells and astrocytes, respectively. In astrocytes the formation of [^{3}H]Sph was completely blocked in both experimental conditions. After a 2-h pulse at 37°C, also radiolabelled glycosphingolipids (mainly [^{3}H]Glc-Cer and [^{3}H]gangliosides) were produced by cultured astrocytes but not by neurons (Fig. 4). The formed [^{3}H]gangliosides were GM3 (92%) and GD3 (8%), the major gangliosides of astrocytes. In the presence of 100 μM chloroquine and at 4°C these molecules were undetectable in both cells.

4. Discussion

The first evidence provided by this work is that primary cultures of neurons (granule cells) and astrocytes from rat cer-

ebellum, are capable to rapidly take up and promptly metabolize exogenous SM. Within a 60 min period of pulse, Cer appeared to be the main metabolite of exogenous SM in both cell types, was produced at an higher rate by granule cells than astrocytes and started being formed early (20 min, possibly less) after exposure to exogenous SM. The formation of ceramide was differently affected in the two cell types by conditions blocking endocytosis or inhibiting lysosomal enzymes activities. In fact, the diminution of formed cer caused by these conditions was only partial (40–17% as percent of controls) in granule cells but almost complete (98.5–90%) in astrocytes. This leads to conclude that the first step of SM degradation occurs essentially at an extralysosomal site (presumably via the plasma membrane-bound SM-ase) in granule cells, and intralysosomally (presumably by the acidic SM-ase) in astrocytes. This interpretation may reflect the fact that the plasma membrane-bound SM-ase is abundant in cultured neurons but absent in glial cells [32]. It would be interesting to assess whether a cytosolic SM-ase occurs in granule cells and concurs to SM degradation.

A further information is that, at the used pulse-times, astrocytes produce Sph from exogenous SM, whereas granule cells do not. In astrocytes Sph formation implies endocytosis and lysosomal processing, since it is completely blocked by incubation at 4°C and chloroquine treatment. A likely explanation for the absence of Sph formation in granule cells is that in these cells the endocytosis-degradation route of SM is scarce and Cer produced at the plasma membrane level does not reach the lysosomes thus avoiding further degradation, at least in the examined period of time. It is surprising that under identical experimental conditions granule cells are able to internalize, via endocytosis, and submit to lysosomal degradation taken up exogenous ganglioside [24], with formation of both Cer and Sph. This may indicate that, after endocytosis, taken up SM is

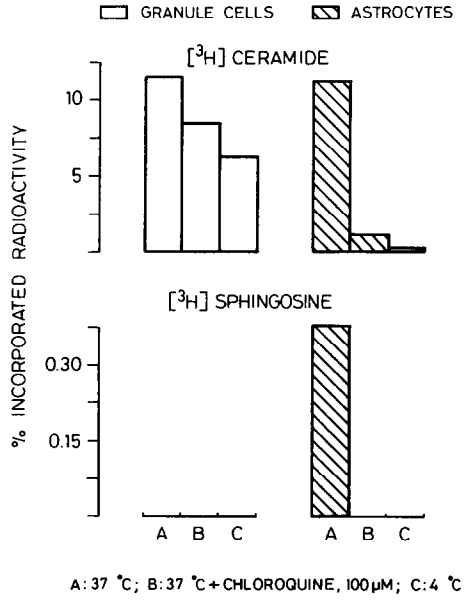

Fig. 3. Incorporation of radioactivity into Cer and Sph by cerebellar granule cells and astrocytes after a 2-h pulse with 2×10^{-6} M [Sph-^{3}H]SM at 37°C, 4°C or at 37°C in the presence of 100 μM chloroquine. The data presented are the mean of three experiments and are expressed as % of incorporated radioactivity referred to controls. S.D. values never exceeded 15% of the mean values.

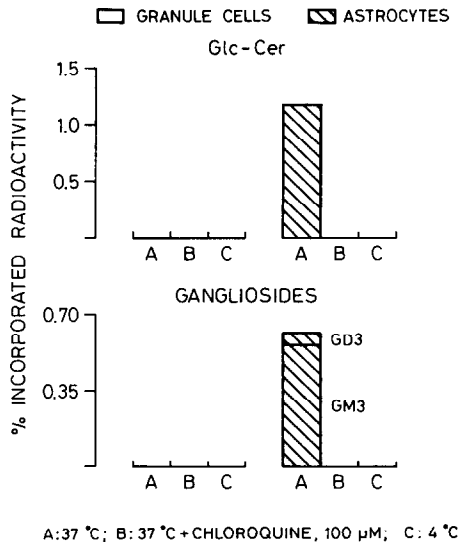

Fig. 4. Incorporation of radioactivity into Glc-Cer and gangliosides by cerebellar granule cells and astrocytes after a 2-h pulse with 2×10^{-6} M [Sph-^{3}H]SM at 37°C, 4°C or at 37°C in the presence of 100 μM chloroquine. The data presented are the mean of three experiments and are expressed as % of incorporated radioactivity referred to controls. S.D. values never exceeded 15% of the mean values.

mainly recycled back to the plasma membrane [4,33], whereas ganglioside is sorted to the lysosomal apparatus.

A final result deserving comment is the formation of glycosphingolipids from exogenous SM metabolism. This event occurs in astrocytes but not in neurons. Under the used experimental conditions glycosphingolipids can be biosynthesized starting from a fragment of SM degradation (Sph, Cer) which escapes further degradation and is reused for biosynthetic purposes at the Golgi apparatus level [23]. In astrocytes, where SM degradation occurs in the lysosomes, the Sph (and/or Cer) produced in the lysosomes serve(s) as the precursor(s). In granule cells, where SM degradation likely takes place in the plasma membrane, formed Cer cannot be easily available at the site of glycosphingolipid biosynthesis.

In conclusion, this work demonstrates that primary cultures of neurons and astrocytes are capable to produce bioactive sphingoids from exogenous sphingomyelin, although through different pathways. In both cell types the formation of sphingoid bioregulators is a relatively rapid process. Therefore, the cellular models presented here appear to be suitable to study the possible implication of sphingolipid-mediated signal transduction in neural functional events, particularly differentiation.

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