Cell type specific localization of sphingomyelin biosynthesis

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Received 22 May 2000; revised 29 June 2000; accepted 29 June 2000

Edited by Guido Tettamanti

Abstract We have studied the incorporation of [^{14}C]serine and of [^{3}H]sphingosine into sphingomyelin in the presence or absence of brefeldin A (BFA) in three different cell types. Administration of BFA (1 μg/ml) to fibroblasts for 24 h increased the incorporation of label into sphingomyelin 1.5–3 fold compared with untreated controls. In contrast, BFA strongly decreased sphingomyelin biosynthesis (4–5 fold) in cerebellar neurons as well as in neuroblastoma cells. The effect of BFA on glycosphingolipid formation, however, was similar in all three cell types studied: an increased labeling of the precursor glycolipids GlcCer, LacCer, GM3 and GD3 was paralleled by a decreased formation of complex gangliosides, GM1, GD1a, GT1b and GQ1b. Our data therefore suggest that in neuronal cells sphingomyelin synthesis, like the formation of complex gangliosides, is localized primarily distal to the BFA block, in a post-Golgi compartment, most probably the *trans*-Golgi network, whereas in fibroblasts sphingomyelin biosynthesis is mainly localized prior to the BFA block, in the Golgi apparatus, as has been shown for LacCer, GlcCer, GM3 and GD3 synthases. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Sphingomyelin; Biosynthesis; Localization; Neuron; Brefeldin A

1. Introduction

Sphingomyelin (SM) constitutes almost 10% of the cellular lipids in mammalian cells and is preferentially concentrated in the outer leaflet of the plasma membrane. Originally it was considered to be mainly a structural element of the plasma membrane, providing a rigid barrier to the environment. Since the discovery of the 'sphingomyelin cycle' as a ubiquitous, evolutionarily conserved signaling system analogous to well known second messenger systems such as the cAMP and phosphoinositide pathways, SM has emerged to the focus of interest in many research laboratories [1,2].

SM, a phosphosphingolipid, is synthesized by the transfer of phosphocholine (the water soluble phospholipid specific head group) from phosphatidylcholine onto ceramide (the hydrophobic sphingolipid specific backbone) [3]. This reaction is catalyzed by SM synthase. Since this enzyme is also able to catalyze the reverse reaction, namely the formation of phosphatidylcholine from SM and diacylglycerol (DAG) [4], it may regulate, in opposite directions, the concentration of two lipid signaling molecules. The roles of DAG and ceramide as anti-apoptotic and pro-apoptotic stimuli, respectively, suggest the biological potential of SM synthase.

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Large efforts have been made to determine the intracellular localization of SM synthase. Subcellular fractionation studies of rat liver have assigned the bulk of SM synthase activity to the lumen of the *cis*-and *medial*-Golgi cisternae, while a minor part, less than 10%, has been localized to the cell surface [5,6]. Subcellular fractionation studies with BHK cells, however, provide evidence that the major site of synthesis of SM is not the Golgi complex but the endocytic recycling pathway [7]. This evidence has been extended in recent years and the new data locate SM synthesis to the *trans*-Golgi network (TGN) of these cells [8]. Contradictory results concerning the localization of SM synthase were also obtained in studies with brefeldin A (BFA), a macrocyclic lactone known to cause Golgi disassembly and its redistribution to the endoplasmic reticulum (ER), thus blocking the vesicular transport from the Golgi complex to the plasma membrane [9]. Based on this property BFA has often been used to map subcellular locations of different glycosyltransferases involved in glycosphingolipid biosynthesis [10–13].

BFA stimulated biosynthetic labeling of SM in CHO cells [14] as well as in rat hepatocytes [15] confirming the Golgi compartment as the major site of SM synthesis. In contrast, BFA induced a considerable decrease of SM labeling with radioactive precursors in cerebellar neurons, favoring a non-Golgi location for SM synthesis [10]. To clarify if these discrepancies are the result of different methodological approaches or rather due to a different subcellular location of SM synthesis in various cell types, in the present study we compared the effect of BFA on sphingomyelin and on glycosphingolipid biosynthesis in postmitotic primary cultured neurons, neuroblastoma cells and fibroblasts. Whereas our data confirm the Golgi compartment as the main place for SM synthesis in fibroblasts, the formation of SM in neurons as well as in neuroblastoma cells is likely to be associated with more downstream elements of the secretory pathway, like the TGN or the plasma membrane.

2. Materials and methods

2.1. Materials

Six day old NMRI mice were obtained from Dr. Brigitte Schmitz from the Institut für Anatomie und Physiologie der Haustiere of the University of Bonn, Germany.

L-[3-^{14}C]Serine (54 mCi/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). [3-^{3}H]Sphingosine (1700 Ci/mol) was obtained according to Sarmientos et al. [16]. Culture media (Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium (MEM) containing Glutamax®) were obtained from Life Technologies, Inc. (Karlsruhe, Germany). DNase was from Roche (Mannheim, Germany). Fetal calf serum (FCS), horse serum and trypsin were supplied from Cytogen (Berlin, Germany). The plastic culture dishes were from Falcon (Heidelberg, Germany). LiChroprep® RP-18 and thin layer silica gel 60 plates were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from Sigma (Deisenhofen, Germany) or Merck.

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PII: S0014-5793(00)01818-4

2.2. Cell culture

Granule cells were cultured from cerebella of 6 day old mice according to the method of Trenkner and Sidman [17]. Cells were isolated by mild trypsinization (0.05%, w/v) and dissociated by repeated passage through a constricted Pasteur pipette in a DNase solution (0.1%, w/v). The cells were then suspended in DMEM containing 10% heat inactivated horse serum and plated onto poly-L-lysine coated 8 cm^2 Petri dishes (6×10^6 cells/dish). Twenty-four hours after plating, cytosine arabinoside (AraC) was added to the medium (4×10^{-5} M) to arrest the division of non-neuronal cells [18]. After 4–5 days in culture, cells were used for metabolic studies.

Human fibroblasts were from the Johanniter Children's hospital, Sankt Augustin, Germany. Cells were cultivated in DMEM supplemented with 10% heat inactivated FCS.

Human neuroblastoma cells (SHSY5Y) were kindly provided by Dr. H. Rösner (Stuttgart, Germany) and were grown to confluence as described [19].

For experiments both fibroblasts and neuroblastoma cells were seeded into 8 cm^2 Petri dishes and grown to confluence.

2.3. Sphingolipid labeling, extraction and analysis

From the cells cultured in 8 cm^2 plastic culture dishes medium was removed and the cells were rinsed two times with MEM. The cells were preincubated for 1 h in the absence or presence of BFA (1 μg/ml) in MEM containing 0.3% horse serum and 1% AraC (cerebellar neurons) or 0.3% FCS (neuroblastoma cells and fibroblasts). Metabolic labeling of sphingolipids (SLs) was performed as described previously [10] by addition of [^{14}C]serine (1 μCi/ml) or [^{3}H]sphingosine (2 μCi/ml) to the culture medium (in which BFA was present or not, as mentioned above). After 24 h, when SL metabolism reached steady state in all three cell types used, cells were washed three times with phosphate buffered saline, harvested and centrifuged at 6000 rpm for 10 min. Total lipids were extracted from cell pellets with 6 ml of chloroform/methanol/water/pyridine (10:5:1:0.1, v/v) for 24 h at 50°C. Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (100 mM) for 2 h at 37°C. The lipid extracts were desalted by reversed phase chromatography on LiChroprep RP18, applied to TLC plates, and chromatographed with chloroform/methanol/0.22% aqueous $CaCl_2$ (60:35:8, v/v); SLs were visualized by autoradiography and identified by their R_F values and enzymatic digestion [10]. Radioactive bands were evaluated by the bio-imaging analyzer Fujix Bas 1000 using software TINA 2.09 (Raytest, Straubenhardt, Germany).

2.4. Protein determination

Cell protein was quantified as described by Bradford [20] using bovine serum albumin as a standard. Before lipid extraction, cell pellets were homogenized in 400 μl of water and aliquots were used for protein determination.

3. Results and discussion

It is well documented that BFA impairs the assembly of ADP ribosylation factor (ARF) and coatomer on Golgi membranes by inhibiting loading of ARF with GTP, thus affecting the budding and formation of transport vesicles and consequently stopping anterograde vesicular transport between Golgi compartments. This causes a redistribution of the Golgi into the ER, and a block from these compartments to the TGN, which is fused with early endosomes [21].

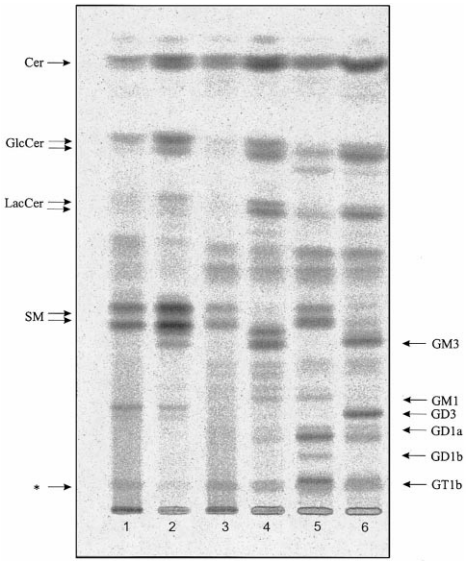

Fig. 1. The effect of BFA on [^{14}C]serine incorporation into sphingolipids of various cell types. Fibroblasts (lanes 1 and 2), neuroblastoma cells SHSY5Y (lanes 3 and 4), and cerebellar neurons (lanes 5 and 6) were incubated for 1 h in the absence (lanes 1, 3 and 5) or presence of 1μg/ml BFA (lanes 2, 4 and 6). Then [^{14}C]serine was added to the culture medium. Cells were harvested after 24 h. Sphingolipids were extracted, isolated, separated by TLC and detected as described in Section 2. The mobilities of authentic sphingolipids are indicated. The terminology for gangliosides (GQ1b, GT1b, GD1b, GD1a, GD3, GM1, GM3) is according to Svennerholm [26]. Other abbreviations are as follows: SM, sphingomyelin; GlcCer, glucosylceramide; LacCer, lactosylceramide; Cer, ceramide. *The nature of this band in fibroblasts and neuroblastoma cells is not known (it is not a ganglioside).

Based on this property BFA uncouples ganglioside biosynthesis beyond GM3, GD3 and GT3 in murine cerebellar neurons [10], CHO cells [11] and chick embryo neural retina cells [13], respectively. We have now followed de novo formation of SM in three different cell types by biosynthetic labeling of cellular SLs with [^{14}C]serine for 24 h in the presence or absence of 1 μg/ml BFA (Fig. 1). To discriminate between the effect of BFA on SM formation via the de novo and salvage pathways, in parallel experiments we used [^{3}H]sphingosine instead of [^{14}C]serine as a labeled precursor for SL biosynthesis (Fig. 2). Note that although sphingosine is not an inter-

Table 1
The effect of BFA on de novo sphingolipid biosynthesis in various cell types

Cell type	[^{14}C]Serine incorporated (% of control)					
	Cer	GlcCer	LacCer	SM	GM3	GD1a
Fibroblasts	415	458	166	267	612	nd
Neuroblastoma cells	441	521	nd	20	1714	nd
Cerebellar neurons	290	390	311	28	1490	30

Cells were preincubated in the absence (control) or presence of BFA (1 μg/ml) for 1 h. [^{14}C]Serine (1 μCi/ml) was then added and the cells were incubated for 24 h. Lipids were extracted, purified and chromatographed as described in Section 2. The data presented were taken from one representative experiment. Two additional experiments were performed and the data obtained were within a range of ±10% of the data given here. The total amount of radioactivity associated with cellular lipids is only slightly changed in the presence of BFA in all three cell types.
nd, not determined since not detectable in controls or both controls and treated cells.
For abbreviations of sphingolipids see legend to Fig. 1.

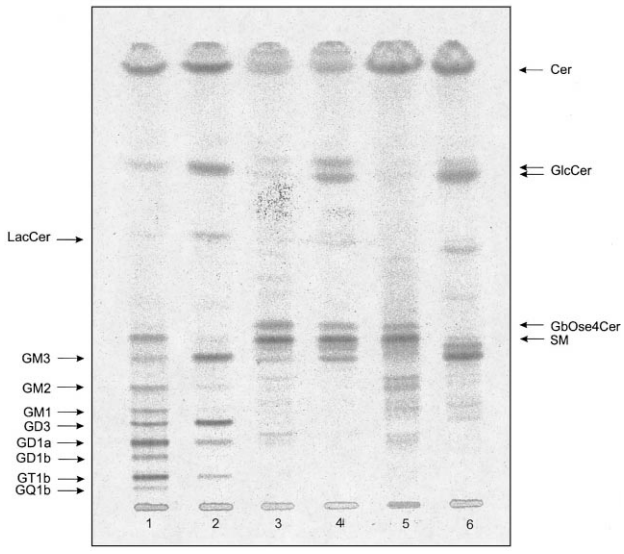

Fig. 2. The effect of BFA on [^{3}H]sphingosine incorporation into sphingolipids of various cell types. Cerebellar neurons (lanes 1 and 2), fibroblasts (lanes 3 and 4) and neuroblastoma cells SHSY5Y (lanes 5 and 6) were incubated for 1 h in the absence (lanes 1, 3 and 5) or presence of 1 μg/ml BFA (lanes 2, 4 and 6). Then [^{3}H]sphingosine was added to the culture medium. Cells were harvested after 24 h. Sphingolipids were extracted, isolated, separated by TLC and detected as described in Section 2. The mobilities of authentic sphingolipids are indicated. For abbreviations see legend to Fig. 1.

mediate in SL biosynthesis but exclusively a catabolic product of ceramide [22], it can be reused for ceramide biosynthesis in a salvage pathway [23].

As shown in Figs. 1 and 2 the SL profile is cell type specific. Whereas in postmitotic, fully differentiated neurons complex gangliosides like GD1a and GT1b are clearly labeled, the SL pattern of neuroblastoma cells resembles that of fibroblasts, complex gangliosides being poorly if at all labeled.

It is clear from Figs. 1 and 2 that BFA induces similar changes in radioactive labeling of all SL species in the three cell types investigated, except for SM. Thus, as shown before in primary cultured neurons [10] and in CHO cells [11], BFA causes a considerable accumulation of label in biosynthetically 'early' SL species, like ceramide, GlcCer, LacCer, GM3 and GD3, with a simultaneous reduction of label incorporation into more complex gangliosides that appear late in the biosynthetic pathway. Only the changes in biosynthetic labeling of SM induced by BFA are cell type specific. In primary cultured neurons as well as in neuroblastoma cells incorporation of label into SM strikingly decreased (down to about 20% of controls), independently of the radioactive precursor used (Tables 1 and 2). In contrast, in fibroblasts an almost threefold and nearly twofold increase of radioactively labeled SM was found when [^{14}C]serine or [^{3}H]sphingosine were used, respectively (Tables 1 and 2).

Thus, BFA increases incorporation of labeled precursors into SM only in fibroblasts, confirming earlier findings in CHO cells [14] and in hepatocytes [15], whereas it causes a striking decrease of SM synthesis in neurons and in neuroblastoma cells. It should be noticed that stimulation of SM biosynthesis observed after administration of BFA is dependent on the radioactive precursor used, being nearly two times higher when [^{14}C]serine is used instead of [^{3}H]sphingosine. Similar observations were made by Brüning et al. [14] in CHO cells. After the addition of BFA the amount of [^{3}H]SM was increased twofold or fivefold when [^{3}H]choline or [^{3}H]C8C8-Cer, respectively, were used to follow SM formation.

Ceramide, the direct biosynthetic precursor of SM, is synthesized on the cytosolic face of ER membranes [24]. In the presence of BFA it is trapped in the newly formed ER/Golgi compartment where it is accessible only for ER/Golgi resident enzymes. Under these conditions only in fibroblasts SM synthase continues to convert ceramide to SM, indicating that this enzyme, like glucosyltransferase and galactosyltransferase I which are responsible for GlcCer and LacCer formation, respectively, as well as the sialyltransferases that synthesize GM3 and GD3, is located in the Golgi compartment [10,11].

Our observation that BFA decreases SM biosynthesis in neurons as well as in neuroblastoma cells indicates that in these cells SM biosynthesis like that of complex gangliosides must be mainly located in a compartment that remains functionally distinct from the ER. This compartment might be the TGN as recently reported for BHK cells [8]. In addition, subfractionation studies of cultured cerebellar neurons located the sialyltransferases responsible for the formation of complex gangliosides to the TGN [25].

The BFA induced block in SM biosynthesis was dramatic (about 80%) but not absolute. The lack of complete inhibition is, however, not surprising and may reflect the dynamic nature of the Golgi/TGN relation. Evidence for a vectorial rather than a clearcut compartmentalization within the Golgi/TGN compartment has already been provided for glycosyltransferases [25]. This may be true also for SM synthase. While the majority of this enzyme may reside *trans* to the BFA block, in a post-Golgi compartment, 10–20% may be located to Golgi elements.

Table 2
The effect of BFA on sphingolipid biosynthesis in various cell types

Cell type	[^{3}H]Sphingosine incorporated (% of control)						
	Cer	GlcCer	LacCer	SM	GM3	GM1	GD1a
Cerebellar neurons	162	307	182	25	304	25	20
Fibroblasts	111	185	nd	170	250	nd	nd
Neuroblastoma cells	105	290	182	20	307	nd	nd

Cells were preincubated in the absence (control) or presence of BFA (1 μg/ml) for 1 h. [^{3}H]Sphingosine (1 μCi/ml) was then added and the cells were incubated for 24 h. Lipids were extracted, purified and chromatographed as described in Section 2. The data presented were taken from one representative experiment. Two additional experiments were performed and the data obtained were within a range of ±10 of the data given here.
nd, not determined since not detectable in controls or both controls and treated cells.
For abbreviations of sphingolipids see legend to Fig. 1.

Acknowledgements: We thank Martina Domgörgen for excellent and reliable cell cultures. This work was supported by the Deutsche Forschungsgemeinschaft SFB 284 and by the German-Israeli Foundation for Scientific Research and Development.

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