

Sphingomyelin is synthesized at the plasma membrane of oligodendrocytes and by purified myelin membranes: a study with fluorescent- and radio-labelled ceramide analogues

Jan P. Vos^{a,**}, M. Luisa Giudici^b, Petra van der Bijl^{a,c}, Paola Magni^b, Sergio Marchesini^b, Lambert M.G. van Golde^a, Matthijs Lopes-Cardozo^{a,*}

^aLaboratory of Veterinary Biochemistry, Utrecht University, Utrecht, The Netherlands

^bDepartment of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy

^cDepartment of Cell Biology, Medical School AZU, Utrecht University, Utrecht, The Netherlands

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Abstract In most cell types sphingomyelin is synthesized predominantly in the *cis-medial* compartments of the Golgi stacks whereas the contribution of the plasma membrane is much lower. The aim of this study was to assess the contribution of both compartments to the synthesis of sphingomyelin in myelinating cells. Therefore, oligodendrocytes from rat spinal cord were incubated in culture with fluorescently- or radiolabelled ceramides, and the effects of a block in the vesicular flow (monensin, brefeldin A, low temperature) on surface synthesis of sphingomyelin were evaluated. The results indicate that $\approx 50\%$ of the sphingomyelin synthase is present at the plasma and myelin membranes of oligodendrocytes.

Key words: Sphingomyelin biosynthesis; Myelin; *N*-Lissamine rhodamine; NBD; Oligodendrocyte (rat)

1. Introduction

Oligodendrocytes are the cells that assemble the huge amounts of myelin membrane to enwrap and insulate the axons in the central nervous system [1,2]. Primary cultures of oligodendrocytes offer an attractive model system to investigate the metabolism and intracellular routing of ceramide derivatives to the myelin membrane which is enriched in sphingolipids.

Mammalian cells generally synthesize sphingomyelin (SM) predominantly at the *cis-medial* Golgi stacks [3,4], but contributions by the plasma membrane [5,6] and of the endocytic recycling pathway [7] have been reported as well. Oligodendrocytes have an intricate network of processes and a very large membrane surface area [2]. Therefore, we wanted to evaluate the

relative activity of SM synthase at the plasma and myelin membranes.

In vivo studies [8,9] have provided indirect evidence that the production of SM occurs at the plasma and/or myelin membranes of myelinating cells. Analysis of radiolabel from lipid precursors injected in the sciatic nerves of rats led Boiron et al. [8] to conclude that the plasma membrane of a Schwann cell, which is the functional analog of the oligodendrocyte in the peripheral nervous system, might be a site of SM synthesis. Furthermore, from the distribution pattern of C₆-NBD-Cer, injected into the ventricles of rat brain, Di Biasi et al. [9] estimated that approx. equal amounts of SM were synthesized at the plasma membrane and the Golgi apparatus of oligodendrocytes.

Sphingomyelin turnover at the plasma membrane is part of signalling pathways in a variety of cell types [10], including cells of neuronal origin [11]. This putative SM cycle, probably operating at the cytosolic face of the plasma membrane [12], could have important implications for the regulation of oligodendrocyte differentiation. The suggestion of a high capacity for SM synthesis at the plasma membrane of myelin producing cells [8,9] and the potential importance of SM in signalling events during myelination, prompted us to investigate the assembly of SM at the plasma membrane of rat spinal cord oligodendrocytes in culture.

2. Experimental

2.1. Materials

UDP-glucose, D-erythro-sphingosine, monensin, D,L- α -hydroxycaproic acid (2-hydroxyhexanoic acid), [1-¹⁴C]hexanoic acid and brefeldin A were purchased from Sigma (St. Louis, USA), D-erythro-[3-³H]sphingosine from DuPont NEN (USA) and C₆-NBD-Cer from Molecular Probes (Eugene, USA). C₆-NBD-labelled lipid reference compounds for high-performance thin-layer chromatography (HPTLC) were kindly provided by Dr. G. Van Meer (Medical School, Utrecht University, The Netherlands). C₁₂-Lrh-lipids were synthesized at the department of Prof. Dr. S. Marchesini (Brescia, Italy; [13]) and radiolabelled ceramides as described elsewhere (Van der Bijl, P. et al., manuscript submitted): C₆-[¹⁴C]-Cer by N-coupling the sphingosine base to [1-¹⁴C]hexanoic acid; C₆-(D- α OH)-[³H]-Cer by N-coupling [³H]sphingosine to D,L-2-hydroxyhexanoic acid and separation of the stereoisomers by TLC (Merck, Darmstadt, Germany).

2.2. Cell culture and incubation with labelled ceramides

Oligodendrocyte-enriched cultures derived from spinal cords of 1-week-old Wistar rat pups were prepared according to [14]. After one day the cultures were shifted to chemically-defined medium (CDM). Experiments were carried out after four days in culture.

*Corresponding author. Fax: (31) (30) 535492.
E-mail: thys@gdl.ruu.nl

**Present address: University of Connecticut Health Center, Department of Microbiology and Program in Neurological Sciences, Farmington, CT 06032-3205, USA. Fax: (1) (203) 679-1239.

Abbreviations: BFA, brefeldin A; BSA, bovine serum albumin; CDM, chemically defined medium; Cer, ceramides; CerGlcT, UDP-glucose:ceramide glucosyltransferase; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; DIC, days in culture; HBSS, Hanks balanced salt solution; HPTLC, high-performance thin-layer chromatography; LRh, *N*-lissamine rhodamine; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PC, phosphatidylcholine; SM, sphingomyelin; SUV, small unilamellar vesicles.

C₁₂-Lrh-Cer or C₆-NBD-Cer were incorporated into small unilamellar egg-PC vesicles (SUV) [15]. Alternatively, labelled ceramides were complexed with BSA immediately before use [16]. Ethanol solutions of C₁₂-LRh-Cer were prepared by dissolving the required amount of dried C₁₂-LRh-Cer in 70% (v/v) ethanol to a final concentration of 100 mM.

The labelled ceramides (SUV, BSA-complex or ethanol solution) were diluted to a final concentration of 20 nmol Cer/10⁶ cells: (i) for long term incubations (24–48 h with SUV); in CDM devoid of BSA; (ii) for short term incubations (3 h): in HBSS/1% (w/v) defatted BSA/20 mM glucose/10 mM Hepes, pH 7.4. Lipid extraction from the cells and medium, separation by HPTLC, lipid extraction from the silica and fluorometry of the fluorescent lipids were performed as described [15]. Incubations were carried out in triplicate. Representative experiments ($n \geq 2$) are shown.

2.3. Purification of myelin and assays for sphingolipid synthesizing enzymes

Myelin was isolated from adult rat brain according to Norton [17]. The crude myelin fraction was subjected to osmotic shocks in distilled water to increase myelin purity and to minimize axolemmal contamination. Myelin purity was determined by measuring the activity of 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). The samples were activated [14] before determining CNPase activity according to [18]. Protein was determined with BSA as standard [19].

SM synthase and UDP-glucose:ceramide glucosyltransferase activities were measured in the same assay system, essentially according to [20]. Briefly, the standard assay mixture (0.5 ml) consisted of 50 mM HEPES-Na (pH 7.4), 5 mM EDTA, 26 μ M C₆-NBD-Cer/174 μ M egg phosphatidylcholine SUV, 5 mM MnCl₂, 500 μ M UDP-glucose and 50–200 μ g of cellular protein. The enzyme reaction was carried out for 30 min at 37°C. Then 1.6 ml of chloroform/methanol (1:2, v/v) was added to the mixture and lipids were extracted by adding 0.5 ml chloroform and 0.5 ml 0.88% (w/v) KCl/10 mM acetic acid. The NBD-labelled sphingolipids were separated by HPTLC and quantified [15].

3. Results and discussion

3.1. Studies with cells in culture

Fig. 1 shows that the accumulation of C₁₂-LRh-SM was arrested upon withdrawal of the fluorescent substrate (egg-PC vesicles containing C₁₂-LRh-Cer) from the medium after a 24 h incubation period. This could result from: (i) a lack of substrate(s); or (ii) limited access of the residual fluorescent ceramide to SM synthase. Option (i) is unlikely, because we have demonstrated previously that only a small fraction of the cell-associated C₁₂-LRh-Cer is metabolized [15] and the intracellular level of C₁₂-LRh-Cer decreased but slightly (<20%) during the chase period (results not shown). Moreover, inspection by confocal scanning laser microscopy showed that the perinuclear region where the Golgi apparatus is situated was labelled intensely already after 3 h of incubation with C₁₂-LRh-Cer SUV

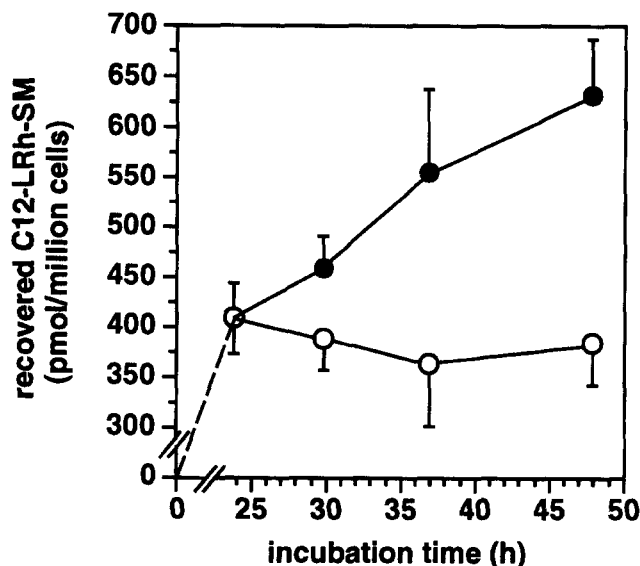


Fig. 1. The production of C₁₂-LRh-SM in oligodendrocytes in primary culture. Cells were incubated with C₁₂-LRh-Cer containing vesicles (20 nmol/10⁶ cells) for 24 h. Thereafter, the cultures were washed twice with HBSS and incubated in medium with (●) or without (○) the C₁₂-LRh-Cer containing vesicles for another 24 h at 37°C. The values represent the total amount of C₁₂-LRh-SM recovered (from the cells + medium). Data (means ± S.E.M.) of a typical experiment ($n = 2$) are shown.

(cf. [22]). At this time point, cell-associated fluorescence amounts to approx. 1.3 nmol/10⁶ cells, and only a small fraction of this (<3%) has been metabolized. Thereafter, products are formed at a higher rate and after 24 h of labelling, the cell-associated fluorescence is approx. 8 nmol/10⁶ cells, of which maximally 1 nmol cells can be attributed to C₁₂-LRh-metabolites [15].

Therefore, we feel confident that the intracellular C₁₂-LRh-Cer concentration is not limiting C₁₂-LRh-SM synthesis. On the other hand, if the major part of C₁₂-LRh-SM is synthesized at the plasma membrane C₁₂-LRh-SM synthesis could be arrested because the egg-PC present in the SUV is a major phosphocholine donor for SM synthesis at the plasma membrane. This possibility was discarded, because the rate of C₁₂-LRh-SM synthesis was the same whether C₁₂-LRh-Cer was added to oligodendrocytes as lipid monomers or incorporated into egg-PC containing SUV. Hence, the most plausible explanation for the

Table 1
Effect of monensin and brefeldin A on SM synthesis in cultured oligodendrocytes

Incubation conditions	Recovery of C ₁₂ -LRh-SM in			Recovery of C ₆ -NBD-SM in		
	Medium ¹	Cells ²	Total	Medium ¹	Cells ²	Total
Control	180 ± 15	11.1 ± 0.9	191	1125 ± 50	96 ± 3	1221
+ Monensin	181 ± 18	11.4 ± 1.1	192	1063 ± 73	174 ± 3	1247
+ BFA	194 ± 32	19.0 ± 0.1	214	895 ± 35	387 ± 16	1282

Oligodendrocytes at 4 DIC were incubated (3 h at 37°C) with BSA-complexes of either C₁₂-LRh-Cer or C₆-NBD-Cer with or without monensin (0.5 μ M) or BFA (1.0 μ g/ml). The cells were preincubated with these drugs for 10 min or 30 min, respectively. Labelled lipids were extracted from the outer leaflet of the plasma membrane by a back-exchange procedure: Incubation of the cells for 15 min at 4°C with 1% (w/v) BSA in HBSS, followed by washing with HBSS. This procedure was performed twice. Control experiments indicated that back-exchanging twice was sufficient to deplete the plasma membrane because no additional labelled lipids could be extracted during a third back-exchange. Data represent the amount of fluorescent SM extracted and are expressed as pmol/10⁶ cells. Values (means ± S.E.M.) of a representative experiment ($n = 2$) are shown.

¹ Labelled SM recovered from the incubation medium and back-exchange medium which were pooled prior to the lipid extraction.

² Labelled SM recovered from the cells after the back-exchange procedure.

Table 2
Distribution of labelled SM in oligodendrocytes after incubation at low temperature

Ceramide precursor	SM synthesized at the plasma membrane (%)
C ₁₂ -LRh-Cer	99.0 ± 9.9
C ₆ -NBD-Cer	55.4 ± 4.9
C ₆ -[¹⁴ C]-Cer	48.8 ± 2.8
C ₆ -(D- α OH)-[³ H]-Cer	52.0 ± 2.6

Oligodendrocytes at 4 DIC were incubated with labelled ceramide complexed to BSA. Labelled ceramides were added to cultured oligodendrocytes in the following concentrations: C₁₂-LRh-Cer and C₆-NBD-Cer (5.0 μ M), C₆-[¹⁴C]-Cer (7.5 μ M; 297 Bq/nmol) and C₆-(D- α OH)-[³H]-Cer (60 nM; 0.2 Bq/nmol). After the incubation for 3 h at 10°C, the outer leaflet of the plasma membrane was depleted by back-exchange (see legend to Table 1). The percentage of plasma membrane synthesis was calculated by dividing the amount of labelled SM recovered from the combined incubation and back-exchange media by the total amount of labelled SM (media + cells). Values are means \pm S.E.M. of triplicate incubations in a representative experiment (with C₁₂-LRh-Cer and C₆-NBD-Cer, $n = 6$; with C₆-[¹⁴C]-Cer and C₆-(D- α OH)-[³H]-Cer, $n = 2$).

observed arrest in C₁₂-LRh-SM synthesis is that C₁₂-LRh-SM is predominantly synthesized at the plasma membrane. Thus, after the removal of the exogenous C₁₂-LRh-Cer, the substrate for C₁₂-LRh-SM synthesis is no longer available and as a consequence C₁₂-LRh-SM does not accumulate any more.

Parenthetically, we note that the appearance of C₁₂-LRh-SM in the medium is not due to the operation of SM synthase in membrane fragments shredded into the medium by the cultured cells. This was verified by control incubations of C₁₂-LRh-Cer in culture medium that had been exposed to oligodendrocytes.

3.2. Effects of monensin, brefeldin A and low temperature incubation

To further discriminate between SM synthesis at the plasma membrane and in the Golgi apparatus we carried out incubations under conditions that block transport of SM, generally considered to be vesicular [23], from the Golgi system to the plasma membrane. Two inhibitors were used: (i) monensin, which inhibits transport of glycoproteins between *medial*- and *trans*- regions of the Golgi apparatus [24] and blocks the ap-

pearance of newly synthesized sphingolipids at the plasma membrane (see [23] for references) without inhibiting SM synthesis from a fluorescent ceramide precursor [25]; (ii) the fungal metabolite brefeldin A (BFA) that causes a reversible disassembly of the Golgi apparatus and inhibits vesicular protein transport along the exocytotic pathway towards the plasma membrane [26,27]. In addition, we carried out incubations at 10°C, a temperature that does not allow vesicular transport of proteins and also blocks sphingolipid transport [25,28].

Table 1 shows that monensin had no and BFA very little effect on C₁₂-LRh-SM synthesis or C₁₂-LRh-SM distribution over cells and medium in 3 h incubations. In contrast, both drugs considerably altered the distribution of C₆-NBD-SM (Table 1). The different behaviour of the two fluorescent probes can be rationalized: C₆-NBD-Cer associates to the plasma membrane of oligodendrocytes, followed by spontaneous bilayer transfer and entry into the cells via monomeric diffusion. Consequently, C₆-NBD-Cer becomes rapidly accessible to SM synthase in the Golgi lumen [25]. On the other hand, C₁₂-LRh-Cer has a longer acyl-chain and a more bulky fluorophore than C₆-NBD-Cer. Importantly, because the half-time for spontaneous lipid movement increases with the chain-length of the acyl-spacer linking the fluorophore to sphingosine [29]. Therefore, we consider it likely that C₁₂-LRh-Cer associates with the outer leaflet of the plasma membrane and cannot reach the luminal face of the Golgi-apparatus as efficiently as C₆-NBD-Cer during a 3 h incubation. For this reason we postulate that C₁₂-LRh-SM is produced predominantly at the plasma membrane; especially during short-term incubations.

This notion is supported by the observed lag time and slow rate of glycosphingolipid synthesis with C₁₂-LRh-Cer as substrate (in contrast to C₆-NBD-Cer) [15]. Also, our observation that monensin and BFA affect C₆-NBD-SM metabolism and transport less in oligodendrocytes than in other cell types, is in agreement with the above hypothesis.

In order to determine the relative contribution of the plasma membrane to the over-all synthesis of SM, several labelled ceramides were incubated with oligodendrocytes at 10°C followed by a back-exchange procedure. Because at 10°C vesicular transport is arrested [28], the amount of labelled SM recovered from the incubation medium should reflect the amount of

Table 3
Activity of SM synthesis in purified myelin. Distribution of plasma-/myelin membrane and Golgi markers

Marker:	SM-synthase ^a	CNPase ^b Myelin	5'-NT ^c PM	APDE-I ^d PM	GP-GalT ^e <i>trans</i> -Golgi	Cer-GlcT ^f <i>cis</i> -Golgi
Homogenate (activity)	(pmol/mg · h) 111 ± 11	(μ mol/mg · h) 595 ± 51	(nmol/mg/h) 2540 ± 73	(nmol/mg/h) 182 ± 7	(pmol/mg/h) 439 ± 10	(pmol/mg/h) 3209 ± 87
Myelin (activity)	235 ± 15	3865 ± 381	7363 ± 111	689 ± 13	164 ± 12	0
(R.S.A.) ¹	2.1	6.5	2.9	3.8	0.4	0
(recovery %) ²	4.2	19.5	5.7	7.4	0.7	0

Myelin was isolated and purified [17] and the following enzymes were assayed: phosphatidylcholine:ceramide phosphocholine transferase (SM synthase [20], a); 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, b; a myelin marker); 5'-nucleotidase (5'-NT, c) and alkaline phosphodiesterase-I (APDE-I, d) as plasma membrane (PM) markers [21]; UDP-glucose:ceramide glucosyltransferase (CerGlcT, f; *cis*-Golgi marker); UDP-galactose:glycoprotein galactosyltransferase (GP-GalT, e; *trans*-Golgi marker). GP-GalT activity was measured by a radioassay. The reaction mixture contained in a total volume of 100 μ l: 50 mM Tris-HCl, pH 7.4; 0.5% (v/v) Triton X-100; 10 mM MnCl₂; 2 mM ATP; 2.0 mg of ovalbumin; 10–50 μ g of cellular protein; 50 μ M of UDP-D-[6-³H]galactose (18.2 Bq/nmol; Amersham Life Science, UK). After 60 min at 37°C, the reaction was stopped with 200 μ l of ice-cold 20% trichloroacetic acid and radioactivity in the washed precipitates was determined. Enzyme assays were carried out in triplicate (means \pm SD) and the experiment was replicated twice with similar results.

¹ R.S.A.: relative specific activity, specific enzyme activity of the purified myelin divided by the specific enzyme activity of the starting homogenate.

² homogenate: the activity found in purified myelin relative to the total activity in the starting homogenate.

SM synthesized at the plasma membrane. Table 2 shows that with all short-chain labelled C₆-Cer precursors approx. half of the newly synthesized SM can be back-exchanged. Therefore, it is plausible that this pool of SM is made in the external leaflet of the plasma membrane.

3.3. SM synthase activity in myelin and at the plasma membrane of cultured oligodendrocytes

Initial attempts to subfractionate cultured oligodendrocytes were in vain because we could not separate plasma membrane markers from Golgi markers. Therefore, pure myelin was isolated, which is an extension of the oligodendroglial plasma membrane. If SM synthase is localized at the plasma membrane, it is likely to be present in myelin as well. Table 3 indicates that SM synthase activity is indeed found in purified myelin. The myelin marker enzyme CNPase [18] was enriched 6-fold over the homogenate. The enrichment in plasma membrane markers [21] 5'-nucleotidase (3×) and alkaline phosphodiesterase (4×) indicates that the myelin fraction contained also a significant amount of plasma membrane. Contrarily, only 0.7% of the total activity of a *trans*-Golgi marker (UDP-galactose:glycoprotein galactosyltransferase) was recovered in the myelin fraction. Moreover, the complete absence of the *cis*-Golgi marker UDP-glucose:ceramide glucosyltransferase [20] indicates that Golgi contamination of the myelin fraction is negligible. This strongly suggests that SM synthase is intrinsic to the myelin membrane and/or plasma membrane fraction.

Our estimate, that about half of the total SM is synthesized at the plasma/myelin membranes (Table 2), corroborates previous estimates from studies *in vivo* [8,9]. Oligodendrocytes might be unique in this respect, because no other cell type has been reported to synthesize such a large proportion of cellular SM at the plasma membrane. More than half of myelin-associated enzymes are involved in lipid metabolism [30] and the data in Table 3 indicate that SM synthase can be added to this growing list of lipid metabolizing enzymes in myelin.

Generally, the bulk of cellular SM resides in the outer leaflet of the plasma membrane and is produced by SM synthase in the lumen of the Golgi stacks [3,4]. Our data on cultured oligodendrocytes indicate that the plasma and myelin membranes can contribute substantially to SM synthesis. It is interesting to note that a distinct pool of SM, probably located at the cytosolic face of the plasma membrane [12], has been postulated to be involved in signal transduction through the operation of the SM cycle [10]. Clearly, the precise topology and physiological roles of SM synthase in myelinating cells remain a topic for future investigation.

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