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Lipid composition of membrane rafts, isolated with and without detergent, from the spleen of a mouse model of Gaucher disease

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ABSTRACT

Biological membranes are composed of functionally relevant liquid-ordered and liquid-disordered domains that coexist. Within the liquid-ordered domains are low-density microdomains known as rafts with a unique lipid composition that is crucial for their structure and function. Lipid raft composition is altered in sphingolipid storage disorders, and here we determined the lipid composition using a detergent and detergent-free method in spleen tissue, the primary site of pathology, in a mouse model of the sphingolipid storage disorder, Gaucher disease. The accumulating lipid, glucosylceramide, was 30- and 50-fold elevated in the rafts with the detergent and detergent-free method, respectively. Secondary accumulation of di- and trihexosylceramide resided primarily in the rafts with both methods. The phospholipids distributed differently with more than half residing in the rafts with the detergent-free method and less than 10% with the detergent method, with the exception of the fully saturated species that were primarily in the rafts. Individual isoforms of sphingomyelin correlated with detergent-free extraction and more than half resided in the raft fractions. However, this correlation was not seen with the detergent extraction method as sphingomyelin species were spread across both the raft and non-raft domains. Therefore caution must be exercised when interpreting phospholipid distribution in raft domains as it differs considerably depending on the method of isolation. Importantly, both methods revealed the same lipid alterations in the raft domains in the spleen of the Gaucher disease mouse model highlighting that either method is appropriate to determine membrane lipid changes in the diseased state.

1. Introduction

Gaucher disease (GD) results from more than 300 known mutations in the gene encoding the lysosomal hydrolase, acid β-glucosidase. This enzyme is a key catabolic hydrolase in sphingolipid metabolism, responsible for cleaving the sphingolipid, glucosylceramide (GC), into its subunits glucose and ceramide. The loss of acid β-glucosidase activity results in GC accumulation in affected cells primarily of the mononuclear phagocyte system (macrophages), especially those in the spleen, liver, lung and bone marrow. The resultant pathology is complex, multi-factorial and progressive, and is associated with hepatosplenomegaly, anaemia, bone disease and central nervous system impairment in the rarer variants [1].

It has been suggested that the primary defect in GC catabolism affects secondary biochemical pathways that are the actual cause of cell and tissue damage [2]. For example, bone disease in a mouse model of GD has been attributed to a defect in osteoblast activity caused by inhibition of protein kinase C by both GC and its lyso derivative [3]. This is perhaps not surprising given that sphingolipid metabolism is a complex network of interdependent events with equally complex regulation, the loss of a lysosomal enzyme activity within sphingolipid metabolism is likely to have significant consequences for the cell.

Previously we have shown that the block in GC catabolism in the lysosome does indeed result in secondary increases in other sphingolipids, and that these increases extend beyond the lysosome [4,5]. This suggests that intracellular trafficking of sphingolipids through the cell to the lysosome is restricted and retrograde transport of GC occurs concurrently with build up in the lysosome [4]. One hypothesis that may explain the sequelae of extra-lysosomal sphingolipid accumulation is the "jamming of the endolysosomal system" with a consequent lack of feedback regulation to

Abbreviations: BMP, bis(monoacylglycero)phosphate; Cer, ceramide; DHC, dihexosylceramide; GD, Gaucher disease; GC, glucosylceramide; LC/ESI-MS/MS, liquid chromatography/electrospray-ionization tandem mass spectrometry; MBS, MES buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; THC, trihexosylceramide.

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the Golgi network for synthesis. This is believed to be mediated by organised membrane microdomains, termed rafts, which are formed by the preferential association of sphingolipids with cholesterol [6]. We have evidence to suggest that the lipid composition of membrane rafts is altered in a macrophage model of GD possibly explaining the altered lipid and protein trafficking seen in this disorder [7,8].

To date, the work addressing the role of raft lipid composition in GD has been restricted to cell models; cell culture artefacts and/or unknown actions of chemical inhibitors on cells cannot be ruled out. Here we have eliminated potential limitations of cell culture by taking a physiological angle and characterised the lipid composition of rafts from an acid β-glucosidase conditional knock-out mouse model in which the resulting phenotype mimics the high levels of visceral GC storage seen in human GD patients [9]. Rafts were isolated from the spleen, the primary site of pathology, to enable an assessment of lipid raft composition within the diseased organ as a whole, rather than isolated cell systems. As far as the variable nature of raft isolation procedures is concerned we used both a detergent and a detergent-free method, and compared the lipid composition of the rafts isolated by both protocols in the diseased and non-diseased state.

2. Materials and methods

2.1. Tissue preparation

All breeding and experimental procedures were performed according to the protocols approved by the institutional Animal Ethics Committee in accordance with the Guidelines of the National Health and Medical Research Council of Australia. The acid β-glucosidase conditional knockout mouse model of GD has been previously described [9]. The spleens from three control (unaffected littermates) and three GD six month old mice were harvested, snap frozen in 98% hexane and stored at −80 °C. Keeping the spleen frozen, approximately 50 mg was cut from the end. After thawing on ice the samples were cut up into smaller pieces (approximately 2 mm) prior to membrane microdomain isolation.

2.2. Isolation of membrane microdomains using a detergent method

Membrane microdomains were extracted from the spleen using the method of Lisanti et al. [10]. Briefly, spleen pieces were placed into a glass Dounce homogeniser with 2 ml MES-buffered saline (MBS) (25 mM MES pH 6.5, 0.15 M NaCl), containing 1% (v/v) Triton X-100 and 1 mM PMSF and homogenised 20 times before being incubated on ice for 30 min. After incubation, homogenates were centrifuged at 425 g for 5 min at 4 °C, after which a 50 μl aliquot of the supernatant was taken for protein determination using the method of Lowry et al. [11]. The remainder of the supernatant was put into the bottom of a 12 ml Beckman (Palo Alto, CA) centrifuge tube and the sucrose concentration was adjusted to 40% (w/v) by the addition of 2 ml 80% (w/v) sucrose in MBS buffer, containing 1% (v/v) Triton X-100 and 1 mM PMSF. The sample was overlayed with 5 ml 30% (w/v) sucrose in MBS buffer, containing 1 mM PMSF and then 3 ml 5% (w/v) sucrose in MBS buffer, containing 1 mM PMSF. Samples were centrifuged at 270,500*g* for 18 h at 4 °C in a SW40 rotor and 1 ml fractions were collected from the top of the gradient.

2.3. Isolation of membrane microdomains using a detergent-free method

Membrane microdomains were isolated from the spleen using a detergent-free method based on the method of Persaud-Sawin et al. [12]. All steps were done on ice or at 4°C. Briefly, spleen pieces were placed into a glass Dounce homogeniser with 1 ml lysis buffer (Tris buffered saline (TBS) pH 8.0, 1% proteinase inhibitor cocktail, 1 mM PMSF, 1 mM $Na_4P_2O_7$, 1 mM Na_2MoO_4, 10 mM NaF, 1 mM NaV, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and homogenised 10–20 times. The homogenate was then sheared 20 times through a 20 gauge needle and centrifuged at 1625*g* for 4 min at 4°C. The supernatant was retained and the pellet was resuspended in 0.5 ml lysis buffer, sheared again and centrifuged at 1625*g* for 10 min at 4°C. The two supernatants were pooled and a 50 μl aliquot was taken for protein determination using the method of Lowry et al. [11]. A portion of the combined supernatant (750 μl) was put into the bottom of a 12 ml Beckman centrifuge tube and the sucrose concentration adjusted to 42.5% (w/v) by the addition of 750 μl 85% (w/v) sucrose in TBS. This was overlayed with 8.5 ml 35% (w/v) sucrose in TBS and then 2 ml 5% (w/v) sucrose in TBS. Samples were centrifuged at 270,500*g* for 18 h at 4°C in a SW40 rotor and twelve 1 ml fractions were collected from the top of the gradient.

2.4. Western blot analysis of membrane microdomains

An aliquot of each membrane microdomain fraction (13 μl) was run on 10% SDS–PAGE gels according to the method of Laemmli [13]. The gels were transferred to PVDF membrane at 30 V for 70 min. The membrane was incubated in block solution (TBS + 0.1% Tween 20 (TBST), 5% (w/v) skim milk pH 7.0) for 1 h at room temperature, washed for 5 min in TBST and then incubated overnight at 4°C in the presence of polyclonal rabbit flotillin-1 (1:1000 in block solution). The membrane was washed 3 × 5 min in TBST and then incubated for 1 h at room temperature in the presence of HRP-conjugated sheep-anti-rabbit immunoglobin (1:5000 in block solution). The membrane was washed 4 × 5 min and developed using the WestFemto ECL blotting system (Thermo Fisher) with detection using the LAS4000 Luminescent Image Analyser (Fujifilm Life Science, Stamford, CT USA).

2.5. Lipid extraction from membrane microdomains

Lipids were extracted from 750 μl of each fraction, using the method of Bligh and Dyer [14] with the inclusion of 400 pmol of the following internal standards: GC 18:1/16:0 (d_3), Cer 18:1/17:0, DHC 18:1/16:0 (d_3), THC 18:1/17:0, PC 14:0/14:0, PE 17:0/17:0, PG 14:0/14:0, PI 16:0/16:0, PS 17:0/17:0 and BMP 14:0/14:0 from Avanti Polar Lipids (Alabaster, AL, USA) and Matreya LLC (Pleasant Gap, PA, USA). The mixture was shaken for 10 min and incubated for 50 min at room temperature. Samples were partitioned by the addition of 950 μl $CHCl_3$ and 950 μl H_2O, shaken for 10 min and centrifuged at 2300*g* for 5 min. The lower hydrophobic phase was transferred to a clean tube and washed with 0.5 ml Bligh–Dyer synthetic upper phase (prepared by mixing 15 ml H_2O with 56 ml $CHCl_3$:CH_3OH (1:2), shaking vigorously for 1 min then adding 19 ml $CHCl_3$ followed by 19 ml H_2O and shaking for another min. The mixture was allowed to stand at room temperature overnight and the top aqueous layer retained for use as the synthetic upper phase), shaken for 10 min and centrifuged at 2300*g* for 5 min. The upper phase was discarded and the lower hydrophobic phase was dried under a gentle stream of N_2 at 40°C.

2.6. Liquid chromatography/electrospray ionisation-tandem mass spectrometry (LC/ESI-MS/MS) quantification of lipids

Prior to analysis the dried extracts were resuspended in 200 μl of CH_3OH containing 5 mM NH_4COOH and individual species of sphingolipids, phospholipids and cholesterol were quantified by LC/ESI-MS/MS in the multiple reaction monitoring mode on a PE Sciex API 4000 triple quadrupole mass spectrometer as previously

described [8], with the inclusion of 18:1/16:0, 18:1/16:1, 18:1/18:0, 18:1/18:1, 18:1/20:0, 18:1/22:0, 18:1/24:0 and 18:1/24:1 species of sphingomyelin (SM). SM used the *m*/*z* product ion of 184 corresponding to the phosphocholine head group and the peak areas were related to the peak area of the PC 14:0/14:0.

3. Results

3.1. Characterisation of membrane rafts from the spleens of GD mice

Flotillin-1 localised the membrane rafts to fractions 3 and 4 with the detergent method and to fractions 2 and 3 with the detergent-free method (Fig. 1). Flotillin-1 showed the same pattern of distribution in the membrane microdomain fractions from the spleens of control mice (data not shown). The distribution of cholesterol also demarcated the membrane rafts to fractions 3 and 4 with detergent isolation (Fig. 2A) and to fractions 2 and 3 with the detergent-free method (Fig. 2B). With the latter there was more cholesterol in the raft fractions than in the soluble domains (fractions 7–12), whereas the converse was true for the detergent method. Based on the combination of flotillin-1 and cholesterol distribution, fractions 3 and 4 were used as the membrane rafts for the detergent method and fractions 2 and 3 for the detergent-free method.

Total GC was determined in each fraction by summing 18:1/16:0, 18:1/20:0, 18:1/22:0, 18:1/24:0 and 18:1/24:1, (Pearson's >0.9) and 73% resided in the membrane raft fractions with both the detergent (Fig. 2C) and detergent-free (Fig. 2D) methods. GC in the rafts isolated from the GD spleens (addition of fractions 2 and 3 for the detergent-free method and fractions 3 and 4 for the detergent method) was 50- and 30-fold above the control spleens, respectively.

3.2. Distribution of lipids across the membrane microdomains in GD spleens

Individual species of Cer, DHC and THC were summed and shown to have a similar distribution with more than half residing in the membrane rafts with both the detergent and detergent-free methods (Fig. 3A). Membrane microdomain profiles for the control spleen tissue were similar to those for GD (data not shown). On the other hand, for the phospholipids, PC, PE, PG, PI, PS and BMP there were differences in distribution across the 12 membrane microdomain fractions between the two methods. Individual species of PC, PE, PG, PI and PS all correlated within their class (Pearson's >0.7), with <10% and >50% of each residing in the membrane rafts in the detergent and detergent-free isolation methods, respectively. However, with detergent the exception was three individual species, PS 16:0/16:0, PC 32:0 and PG 32:0 that did not correlate with the other species in their class and approximately 55% of each resided in the raft fractions (Fig. 3B). For the phospholipid, BMP, all species correlated (Pearsons >0.7) and only 5% of BMP was found in the raft fractions with the detergent method but with detergent-free isolation, the raft fractions contained 51% BMP (Fig. 3C).

The distribution of SM with the detergent-free method was similar to the other phospholipids with all species correlating (Pearson's >0.7) and 54% residing in the raft fractions. However, with detergent extraction SM 18:1/20:0, 18:1/16:1, 18:1/18:1 and 18:1/22:0 correlated (Pearson's >0.7), and 18:1/16:0, 18:1/18:0, 18:1/24:0 and 18:1/24:1 correlated (Pearson's >0.7), were summed, and are referred to as Group 1 and Group 2, respectively. Fig. 3D shows that the two groups of SM differed in their distribution; only 11% of SM Group 1 was found in the raft fractions compared with 57% of SM Group 2. The control spleen tissue showed the same distribution of phospholipids as the GD spleen (data not shown).

3.3. Accumulation of lipids in membrane rafts

To investigate the lipid alterations in the spleen rafts from mice with GD compared to controls, the lipid concentrations in fractions 3 and 4 for detergent, and 2 and 3 for detergent-free were summed, excluding those that did not correlate. Fig. 4A shows that although statistical significance was not achieved for the detergent method, Cer, DHC and THC increased in the GD spleen rafts compared to controls. These same lipids were significantly increased in GD spleen rafts with detergent-free isolation (Fig. 4B). Significant increases of up to 6-fold in the GD spleen rafts were also seen for SM Group 1, PC (excluding PC 32:0), PE, PG (excluding PG 32:0) and BMP with detergent isolation (Fig. 4C). Similarly, significant increases of up to 5-fold in SM, PE, PG and BMP were observed with the detergent-free method (Fig. 4D). Importantly, there was no difference in the lipid composition of membrane rafts with either the detergent or detergent-free method.

4. Discussion

In this work, we have shown that there is no difference in the distribution of Cer, GC, DHC and THC across the membrane microdomains with a detergent or detergent-free membrane raft isolation method. These sphingolipids reside primarily in the membrane rafts and were shown to be increased in the spleens of the GD mice regardless of the extraction method used. This is a characteristic feature of raft domains, as sphingolipids, which generally have saturated or mono-unsaturated fatty acids, preferentially interact with cholesterol and are able to pack together at a higher density than phospholipids, resulting in the liquid ordered phase [6]. The detergent-free method produced less variable results compared with the detergent method as Fig. 4A and B shows that although the elevation pattern of Cer, DHC and THC was present in the GD spleens with both methods, only statistical significance was achieved with the detergent-free method. Additionally, Fig. 2 shows that the amount of cholesterol and GC recovered in the rafts using the detergent-free method was greater than

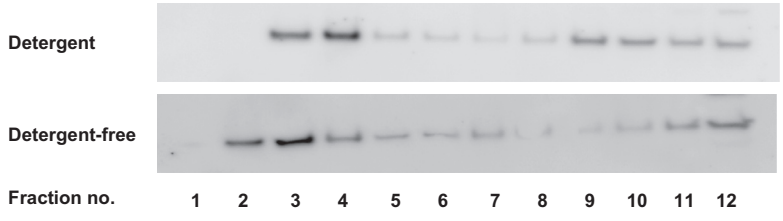

Fig. 1. Flotillin-1 distribution across the membrane microdomain fractions in mouse spleens. Membrane microdomains were isolated from six month old GD mice using a detergent or detergent-free method and the presence of the raft marker, flotillin 1, by Western blot is shown.

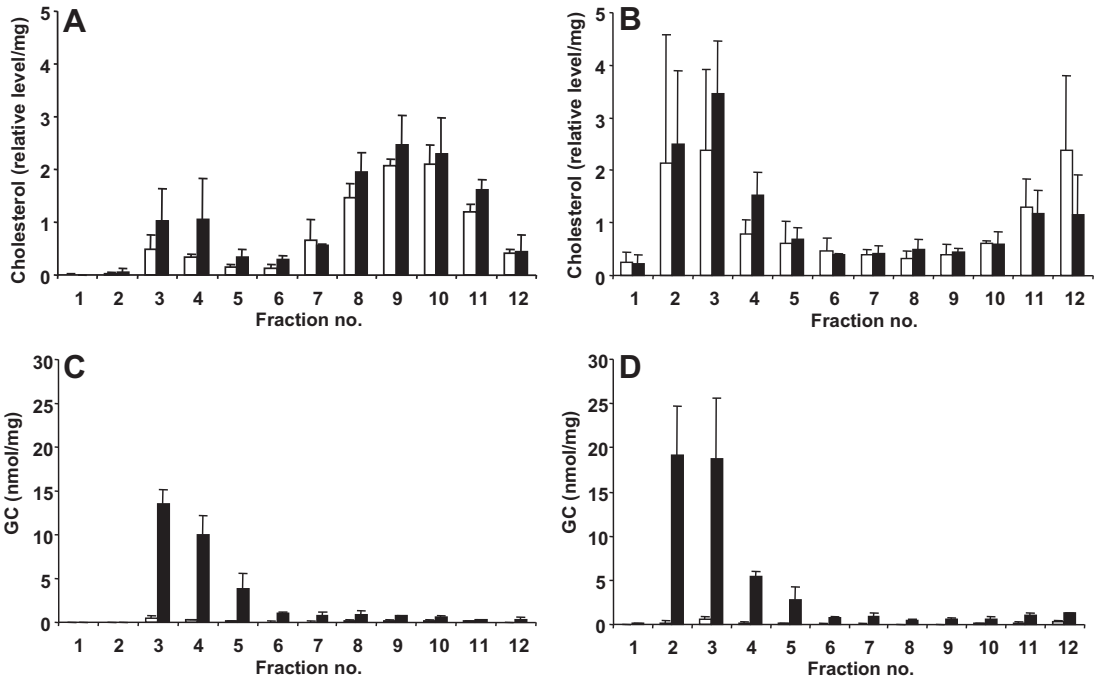

Fig. 2. Cholesterol and GC distribution across the membrane microdomain fractions of control and GD spleens. Cholesterol is expressed as a ratio of the 2:0 cholesteryl ester to the 17:0 internal standard per mg of total protein and is shown for control (open bars) and GD (filled bars) spleens with the detergent (A) and detergent-free (B) isolation methods. GC is reported as nmol per mg of total protein for control (open bars) and GD (filled bars) spleens for detergent (C) and detergent-free (D) extractions. Results are expressed as the mean and standard deviation ($n = 3$).

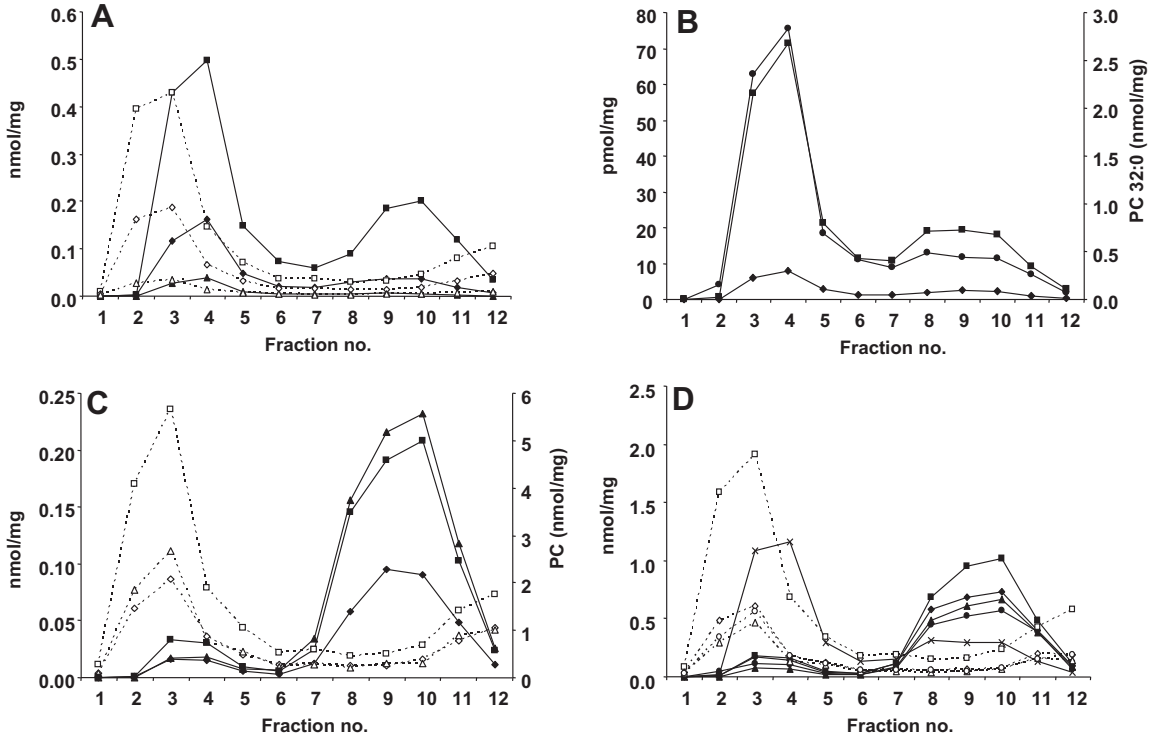

Fig. 3. Total lipid distribution across the membrane microdomain fractions. Individual species of Cer (squares), DHC (diamonds) and THC (triangles) were summed in each of the membrane microdomain fractions to give total amounts and are shown in A. Concentrations of PC 32:0 (squares), PS 16:0/16:0 (circles) and PG 32:0 (diamonds) across the membrane microdomain fractions were graphed separately for detergent extraction (B). Individual species of PC (squares), PG (diamonds) and BMP (triangles) were summed in each of the membrane microdomain fractions to give total amounts (C). Individual species of SM (detergent-free squares, detergent group 1 squares, detergent group 2 crosses), PE (diamonds), PI (triangles) and PS (circles) were summed in each of the membrane microdomain fractions to give total amounts (D). Results are reported as nmol/pmol of lipid per mg of total protein loaded onto the gradient prior to fractionation. Detergent method shown as solid lines and detergent-free method denoted as dashed lines.

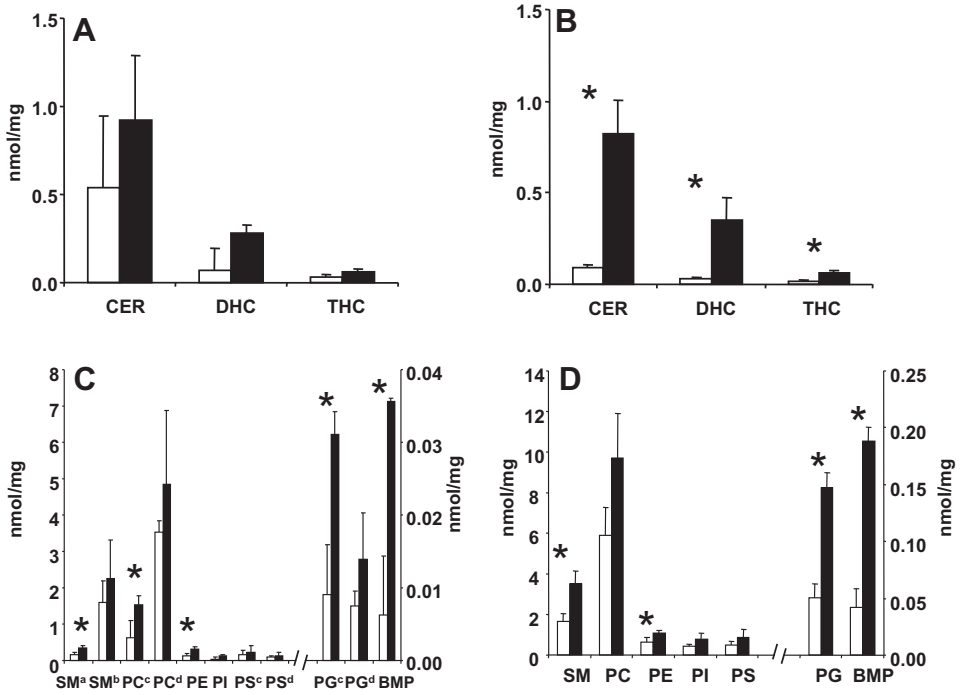

Fig. 4. Lipid accumulation in membrane rafts from control and GD spleens. Total concentrations (nmol per mg of total protein loaded onto the gradient prior to fractionation) of Cer, DHC and THC from the detergent (A) and the detergent-free isolation method (B), as well as SM, PC, PE, PG, PI, PS and BMP from the detergent (C) and detergent-free isolation method (D) are shown for control (open bars) and GD (filled bars) membrane rafts. Results are expressed as mean and standard deviation ($n = 3$). *Significant at $p < 0.05$ (Student's t-test). [a]SM Group 1; 18:1/20:0, 18:1/16:1, 18:1/18:1 and 18:1/22:0 [b]SM Group 2; 18:1/16:0, 18:1/18:0, 18:1/24:0 and 18:1/24:1[c]PC, PS and PG species that correlated with one another, with the exception of [d]PC 32:0, PS 16:0/16:0 and PG 32:0 which did not correlate with the other species within their class.

the detergent method, suggesting that the detergent was less effective in enriching these lipids.

The real difference between the two extraction methods lay with the distribution of the phospholipids. As expected, detergent extraction excluded the majority of the phospholipids from the raft domains with the exception of the shorter acyl chain length (16:0) e.g., PS 16:0/16:0 and fully saturated fatty acid species e.g., PC 32:0 and PG 32:0 that presumably are the 16:0/16:0 [15] but we were not able to confirm this using the mass spectrometry measurement. The nature of these phospholipid fatty acid side chains supports the notion of the raft concept as it allows them to pack tightly with the saturated acyl chains of sphingolipids, and with cholesterol, resulting in the liquid ordered domain [16]. Excluded from these raft domains are the unsaturated phospholipids which, even in the presence of cholesterol, remain more fluid and exist in the rest of the membrane [17]. These results are in agreement with previous work reporting the majority of phospholipids reside in the detergent soluble domains with the exception of the short acyl chain, fully saturated species that are present in the rafts [7,8]. The opposite effect was seen with the detergent-free method of extraction as at least half of the phospholipids were present in the raft domains and there was no segregation of the fully saturated species. This suggests that the use of detergent (Triton X-100) partitions the fully saturated phospholipids away from the rest of the phospholipids, into what are often referred to as detergent resistant membranes. Detergent is used to disrupt the lipid-lipid interactions in the membranes and relies on the principle that the liquid ordered membrane fraction is tightly packed and therefore resists detergent extraction [18]. This tight packing must therefore involve the shorter fully saturated fatty acid phospholipid species and exclude the other species, when Triton X-100 is used. Other detergents have also been used to isolate rafts and the different detergents are reported to have varying selectivities for enriching particular lipid types [19].

SM also showed the same distribution as the other phospholipids with the detergent-free method but with detergent some of the SM species distributed differently. Not all the SM species correlated together but correlated in two groups that did not bear any resemblance to the degree of unsaturation or acyl chain length of the fatty acids. The explanation for the differences in the individual species of SM is not clear, but it may be due to the efficiency of detergent extraction to partition the species of SM. As one of these groups (SM Group 2) distributed similarly to the SM distribution using the detergent-free method, the differences in SM distribution between the two methods was not as great as for the other phospholipids (Fig. 3). The other phospholipid analysed was BMP; being a lysosomal phospholipid its presence demonstrated that the rafts isolated here were not restricted to the plasma membrane. BMP has previously been shown to be elevated in GD [20] and here we show, despite the numerous different fatty acid species measured, the distribution is the same as its presumed synthetic precursor PG, residing primarily in the rafts in the detergent-free method and in the non-raft domains with detergent extraction (Fig. 3C).

In conclusion, this side-by-side comparison shows membrane rafts isolated with a detergent method or detergent-free method do not differ in their sphingolipid composition. The difference between the two methods is only apparent when comparing the phospholipid distribution, suggesting that the detergent method enriches a subset of rafts. Furthermore, despite raft extraction on whole spleen homogenates, representing a mixed cell population, the lipid changes and distributions were remarkably similar to a previously reported THP-1 macrophage cell line [7,8]. Two different types of raft domains have been isolated by the two methods reported here, but neither necessarily reflects microdomains in

the native membrane. Nonetheless, the alterations in the lipids as a consequence of GD were the same with both methods, illustrating either are suitable tools to assess membrane lipid differences between the disease and non-disease state.

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