Glucosylceramide modulates membrane traffic along the endocytic pathway

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Abstract Glycosphingolipids are endocytosed and targeted to the Golgi apparatus, but are mistargeted to lysosomes in numerous sphingolipidoses. Substrate reduction therapy utilizes imino sugars to inhibit glucosylceramide synthase and potentially abrogate the effects of storage. Gaucher disease is a hereditary deficiency in glucocerebrosidase leading to glucosylceramide accumulation; however, Gaucher fibroblasts exhibited normal Golgi transport of lactosylceramide. To better understand the effects of glycosphingolipid accumulation on intracellular trafficking and the use of imino sugar inhibitors, we studied sphingolipid endocytosis in fibroblast and macrophage models for Gaucher disease. Treatment of fibroblasts or RAW macrophages with conduritol B epoxide, an inhibitor of lysosomal glucocerebrosidase, resulted in a change in the endocytic targeting of lactosylceramide from the Golgi to the lysosomes. Co-treatment of macrophages with conduritol B-epoxide and 12–25 μM N-butyldideoxygalactononjirimycin, an inhibitor of glycosphingolipid biosynthesis, prevented the mistargeting of glucosylceramide to the lysosomes and restored trafficking to the Golgi. Surprisingly, higher doses (>25 μM) of NB-DGJ induced targeting of lactosylceramide to the lysosomes, even in the absence of conduritol B-epoxide. These data demonstrate that both increases and decreases in glucosylceramide levels can dramatically alter the endocytic targeting of lactosylceramide and suggest a role for glucosylceramide in regulation of membrane transport.—Silence, D. J., V. Puri, D. L. Marks, T. D. Butters, R. A. Dwek, R. E. Pagano, and F. M. Platt. Glucosylceramide modulates membrane traffic along the endocytic pathway. J. Lipid Res. 2002, 43: 1837–1845.

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The glycosphingolipidoses are a group of inherited metabolic diseases in which glycosphingolipids (GSLs) accumulate due to their impaired lysosomal breakdown. The majority are autosomal recessive diseases resulting from mutations in the genes that encode the glycosphingolipid hydrolases, which sequentially degrade GSLs in the lysosome. Impaired GSL hydrolysis leads to GSL accumulation in various cells and tissues of affected individuals. However, the mechanism by which the abnormal accumulation of GSLs leads to pathology is not known. The most prevalent glycosphingolipid storage disorder is Gaucher disease (type 1), which is due to defective glucocerebrosidase, an enzyme that breaks down lysosomal glucosylceramide (GlcCer). Type 1 Gaucher disease is an unusual disorder, because although the enzyme deficit is ubiquitously present in all cell types, only one cell lineage stores GlcCer, the tissue macrophage. Its storage burden is not derived from de novo synthesis but from the ingestion of apoptotic and senescent cells. Residual enzyme activity is therefore sufficient to fully catabolise GlcCer in all other cell types, the macrophage’s storage burden is acquired indirectly.

Substrate reduction therapy utilizes imino sugar inhibitors of GlcCer synthase, which catalyzes the first glycosylation step in GSL formation to reduce the rate of GSL synthesis and abrogate the effects of lipid storage. This approach has been applied to the treatment of mouse models of Sandhoff and Niemann-Pick C diseases (1, 2), and in clinical trials of Gaucher disease (3, 4). To better understand the use of imino sugar inhibitors for the treatment of the glycosphingolipidoses, we have studied the endocytosis and intracellular targeting of GSLs in Gaucher fibroblasts and macrophages and

Abbreviations: BODIPY-LacCer, N-[5-(5,7-dimethylborondipyrromethenedifluoride)-1-penanoyle]-lactosylphingosine; CBE, conduritol B epoxide; GalSph, galactosylphingosine (psychosine); GlcCer, glucosylceramide; GlcSph, glucosylphingosine; GSL, glycosphingolipid; NB-DHJ, N-butyldideoxyhomonojirimycin; NB-DGJ, N-butyldideoxygalactonojirimycin; NB-DMJ, N-butyldideoxymannojirimycin; PDMP, N,N-threo-1-phenyl-2-decanoylamino-3-morpholinole-1-propanol; SLSD, sphingolipid storage disease.

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examined the effects of the imino sugar inhibitors on these processes.

Changes in endocytic sorting have been shown to occur in most sphingolipid storage disease (SLSD) fibroblasts using a fluorescent analogue of lactosylceramide N[5,5,7-dimethylboryldipyrromethenedifluoride]-lactosylsphingosine (BODIPY-LacCer) (5–8). In normal fibroblasts, this lipid is internalized from the plasma membrane by a clathrin-independent (caveolar-related) mechanism and subsequently targeted to the Golgi apparatus, while in SLSD cells the fluorescent lipid analogue accumulates in late endosomes and lysosomes (5–8). In the SLDs, aberrant intracellular targeting of LacCer has been linked to intracellular cholesterol levels. However, the mechanism(s) by which SL and cholesterol accumulation induce changes in trafficking remain unknown, although it has been demonstrated that altered trafficking in SLSD cells can be reversed by depleting intracellular cholesterol (7). This suggests that both the accumulation of endogenous SLs and alterations in intracellular cholesterol play a role in modulating lipid trafficking along the endocytic pathway. Importantly, SL binding toxins also show altered trafficking, suggesting that the sorting of endogenous GSLs is also perturbed in these diseases (8). It is hypothesised that increases in lysosomal cholesterol can mediate changes in the trafficking of membrane lipids and proteins either through gross changes in intraluminal membrane fluidity or through coalescence of cholesterol-sphingolipid microdomains (9, 10). Changes in cholesterol could therefore lead to impairment of the function of important signalling proteins and form the basis of the pathology of SLDs at the cellular level.

While altered intracellular trafficking of GSLs has been observed in nine different SLSD cell types, no altered trafficking is seen in Gaucher fibroblasts (6, 7). However, this could be due to the fact that only macrophages store this disorder. Alternatively, the normal trafficking in Gaucher disease could imply that GlcCer is unique in not inducing altered trafficking when stored. It is not known in any of these diseases whether altered trafficking directly causes pathological changes in cells ultimately causing the clinical phenotype.

In the present study, we pharmacologically manipulate the intracellular levels of endogenous GlcCer and determine the effect on intracellular trafficking of BODIPY-LacCer in human fibroblasts and RAW macrophages. Gaucher fibroblasts did not accumulate GlcCer under basal conditions due to residual enzyme activity; however, induction of GlcCer storage with conduritol B epoxide (CBE) induced altered targeting of BODIPY-LacCer to endosomes/lysosomes in both fibroblasts and macrophages, and also led to changes in cholesterol distribution. These data suggest that the endocytic sorting of lipids is altered in Gaucher macrophages, which are known to store GlcCer. Unexpectedly, we also found that an ~50% decrease in GlcCer levels induced by inhibition of ceramide glucosyltransferase with high concentrations of the imino sugar, N-butyldexygalactonojirimycin (NB-DGJ), or 2,3-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) resulted in lysosomal accumulation of BODIPY-LacCer and increased intracellular cholesterol levels. Normal BODIPY-LacCer transport could be restored by the addition of glucosylsphingosine (GlcSph), which partially replenished cellular GSLs. These data suggest that GlcCer plays a key role in the endocytic sorting of both GSL and cholesterol in macrophages. Furthermore, a proven therapy for type 1 Gaucher disease (3) prevents the altered trafficking, suggesting that this mechanism may form the basis for the cellular pathology in this disease.

**EXPERIMENTAL PROCEDURES**

**Materials**

[1-14C]galactose was from Amersham Pharmacia Biotech (Rainham, Essex, UK) and [14C]palmitate from ICN (Thame, Oxfordshire, UK). Tissue culture media and supplements were from Gibco (Paisley, Renfrewshire, Scotland, UK). LysoTracker red and BODIPY-LacCer were from Molecular Probes (Leiden, Netherlands). NB-DGJ and Conduritol B-epoxide were from Toronto Research Chemicals (Ontario, Canada). GlcSph and galactosylsphingosine (GalSph) were from Matreya (State College, PA).

**Cell culture**

Normal and SLSD fibroblasts were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository and were cultured as described (6). RAW 264.1 mouse macrophages were obtained from the ECACC (Porton Down, UK) and maintained at a density of 5 × 10^5 cells/ml in RPMI supplemented with 10 mM glutamine, 50 U/ml penicillin/streptomycin and 10% FCS.

**Treatment of cells with drugs**

RAW cells (12.5 × 10^6 cells/well) or fibroblasts (~10^6 cells/well) were labelled and grown for 48 h in the presence of 20 μCi of [14C]palmitate or 2.5 μCi [14C]galactose. Galactose labelled cells were decylated in methanolic NaOH and lipids were separated by TLC (CHCl₃/CH₃OH-0.22% CaCl₂ 65:35:8; v/v/v) (11) and quantitated by phosphorimaging. Cold lipids were quantitated by densitometry using National Institutes of Health image software by comparison with known standards. RAW cells were treated with 10 μM PDMP, 50 μM CBE, or 0–100 μM imino sugar. NB-DGJ and CBE were dissolved in water at 1000× concentration, 0.2 μm filtered and stored at −20°C. PDMP, GlcSph, and GalSph were dissolved at 1000× concentration in ethanol, whereas GlcCer was dissolved at 500× concentration in DMSO-ETOH (1:1, v/v). None of these treatments lead to significant increases in cell death as judged by propidium iodide staining and flow cytometric analysis.

**Cholesterol quantitation**

10^7 RAW cells were harvested, resuspended in 1 ml of PBS, and 100 μl of the suspension removed for protein determination using a commercial kit (Pierce, IL). Following lipid extraction, the lower phase was dried under N₂ and neutral lipids separated by TLC (CHCl₃-C₆H₆-O₂C₃H₅-CH₂-OOH 65:15:1; v/v/v) and iodine stained overnight. Cholesterol was quantitated by densitometry using cholesterol standards. Detergent insoluble pellets were generated according to Ledesma (12). Briefly, cells were extracted for 1 h at 4°C in TBS (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA and a mixture of antiproteases) containing 1% Triton X-100. Insoluble material was pelleted by a 10 min centrifugation at 15,000 g at 4°C.
Fluorescence microscopy.

RAW cells or human fibroblasts were labelled with BODIPY-LacCer by pulsing with either BODIPY-LacCer in 1% serum for 30–60 min, removing cell surface fluorescence by washing 3 times 1 min with 10% serum and chasing for 60–90 min depending on the cell type (5). In double label experiments, cells were incubated with 200 nM Lysotracker red for the final 15 min of the incubation. Filipin staining was performed as described (13). Fluorescent cells were observed using a Zeiss Axioplan 2 fluorescence microscope. Lysotracker red was excited at 546 nm and fluorescence observed at >590 nm; BODIPY-LacCer was excited at 450–490 nm and viewed at >520 nm; filipin was excited at 360 nm and viewed at 460 nm. Images were collected using a charge-coupled device camera and analysed using Axiovision software (Image Associates Thame, UK).

RESULTS AND DISCUSSION

GSL storing fibroblasts accumulate BODIPY-LacCer in endocytic structures

When normal human skin fibroblasts are pulse-labeled with BODIPY-LacCer, the fluorescent lipid is targeted primarily to the Golgi complex as judged by colocalisation with mannosidase II and TGN 38 (14). In fibroblasts isolated from patients with Fabry disease and most other GSL storage disorders, fluorescence accumulates in punctate endocytic structures (Fig. 1) (6). One exception, however, is Gaucher fibroblasts that show normal trafficking of BODIPY-LacCer (Fig. 1). We suspected this might be due to the lack of substantial GlcCer storage in Gaucher fibroblasts, since storage of GlcCer is largely restricted to macrophages, at least in type 1 Gaucher disease, since they have an increased burden of GlcCer due to their phago-}

Fig. 1. Endocytic sorting of N-[5-(5,7-dimethylborondipyrromethene)difluoride]-1-penanoyl-lactosylphingosine (BODIPY-LacCer) in normal, sphingolipid storage disease (SLSD), and conduritol B epoxide (CBE) treated human skin fibroblasts. Representative images of fibroblasts pulse-chased with BODIPY-LacCer and viewed under the fluorescence microscope. Where indicated, normal fibroblasts were incubated with 50 μM CBE for 24 h. Scale bar 10 μm.

TABLE 1. Glycosphingolipid composition of human fibroblasts from patients with GSL-storage diseases

| Glycosphingolipid | Normal | Gaucher I | Gaucher II | Tay-Sachs | Fabry |
|-------------------|--------|-----------|------------|-----------|-------|
| GlcCer            | 4 ± 1  | 5 ± 1     | 3 ± 1      | 4 ± 1     | 5 ± 1 |
|Gb3               | 21 ± 4 | 26 ± 3    | 22 ± 4     | 20 ± 2    | 33 ± 4* |
|GM3               | 22 ± 6 | 18 ± 2    | 17 ± 2     | 24 ± 3    | 18 ± 3 |
|GM2               | 4 ± 1  | 5 ± 1     | 3 ± 1      | 9 ± 1     | 3 ± 1 |
|GM1               | 3 ± 1  | 4 ± 1     | 5 ± 2      | 5 ± 1     | 3 ± 1 |
|GD3               | 7 ± 3  | 7 ± 2     | 9 ± 3      | 10 ± 5    | 5 ± 2 |

Human fibroblasts were grown to confluence with [14C]galactose for 48 h. Lipids were extracted and quantitated. Of the 2.5 μCi added, 128 ± 21 nCi (mean ± SE; n = 4) were incorporated into lipid. Of this, 69 ± 4% was incorporated into GSL with the balance in phosphatidylcholine and neutral lipids. No consistent changes could be detected in the upper and lower bands of sphingolipids as they appear on TLC. Results are from four independent experiments and are expressed as percentage of total incorporation into lipids ± SEM.

* P < 0.05.
the accumulation of BODIPY-LacCer in punctate cytoplasmic structures (Fig. 2A). However, 24 h treatment with CBE caused GlcCer levels to increase from 1.2 to 2.2% of total lipid and this was all that was necessary to observe the redirection of BODIPY-LacCer trafficking (Fig. 2A). Co-localisation with lysotracker red demonstrated that CBE treated cells were accumulating BODIPY-LacCer in the lysosome (Fig. 2B). Treating macrophages and fibroblasts with 40 μM GlcCer for 48 h also induced a similar punctate distribution (results not shown). Moreover, the relative distributions of lysotracker red and NBD-ceramide (a marker for the Golgi) (18) were similar in control and CBE treated cells (Fig. 2C). It is interesting to note that even this subtle elevation in GlcCer, well below that observed in symptomatic Gaucher patients (19), was sufficient to induce an alteration in glycolipid trafficking.

Substrate reduction therapy using imino sugar inhibitors of GlcCer synthase have recently been used for the treatment of Gaucher disease (3, 4). It was therefore interesting to see whether the effect of CBE on lipid trafficking to the lysosome could be prevented by coadministration of the imino sugar inhibitor, NB-DGJ (11, 20). The punctate intracellular staining of BODIPY-LacCer induced by CBE could be blocked by coadministration of 12–25 μM NB-DGJ leading to Golgi-like perinuclear staining (Fig. 2A). However, surprisingly, higher concentrations of NB-DGJ (>25 μM) failed to reverse this effect and a punctate intracellular staining pattern persisted (Fig. 2A) though Golgi morphology was normal (Fig. 2C). GlcCer has also been implicated in vesicular sorting in melanoma cells (21).

GlcCer depletion alters sorting of BODIPY-LacCer to punctate endocytic structures

Administration of 100 μM NB-DGJ alone for 24 h caused lysosomal targeting of BODIPY-LacCer as was observed with GlcCer storage (Fig. 3B vs. 2B). Similar results were also obtained with PDMP (Fig. 3C), suggesting that both decreases and increases in GlcCer levels induce altered trafficking of BODIPY-LacCer from the Golgi to the lysosome. The effect of NB-DGJ on BODIPY-LacCer en-
Endocytic sorting was dose-dependent (Fig. 3B) and occurred at concentrations of NB-DGJ above 25 μM. Redirection of BODIPY-LacCer trafficking was specific to imino sugars, which inhibit GSL biosynthesis. Chemically related non-GSL biosynthesis inhibitors such as non-alkylated DGJ (DGJ), N\textsubscript{-}butyl deoxyhomonojirimycin (NB-DHJ), and N\textsubscript{-}butyl deoxymannojirimycin (NB-DMJ) do not inhibit GlcCer biosynthesis (11) and had no effect on BODIPY-LacCer trafficking within the 24 h time frame of the experiment (Fig. 3C). PDMP, a chemically unrelated inhibitor of ceramide glucosyltransferase (22), had a similar effect to NB-DGJ, suggesting that changes in BODIPY-

### Table 2. Lipid composition of RAW macrophages treated with NB-DGJ and CBE for 24 h

| NB-DGJ  | 0 μM   | 6 μM   | 12 μM  | 25 μM  | 50 μM  | 100 μM | CBE |
|---------|--------|--------|--------|--------|--------|--------|-----|
| NL      | 3 ± 0.3| 3 ± 0.3| 3 ± 0.3| 3 ± 0.4| 3 ± 0.4| 3 ± 0.5| 3 ± 0.3|
| GlcCer  | 1.2 ± 0.2| 1.1 ± 0.1| 0.9 ± 0.2| 0.9 ± 0.2| 0.6 ± 0.1\*| 0.6 ± 0.1\*| 2.2 ± 0.3\*|
| PtdEm   | 31 ± 1 | 31 ± 1 | 30 ± 1 | 29 ± 1 | 30 ± 1 | 31 ± 1 | 30 ± 1 |
| PtdCho  | 40 ± 4 | 40 ± 3 | 43 ± 2 | 43 ± 2 | 43 ± 2 | 41 ± 3 | 42 ± 2 |
| PS/PI   | 7 ± 1  | 7 ± 1  | 7 ± 1  | 7 ± 1  | 7 ± 1  | 7 ± 1  | 7 ± 1  |
| SM      | 12 ± 1 | 12 ± 2 | 12 ± 1 | 13 ± 1 | 15 ± 1 | 15 ± 1 | 12 ± 1 |
| GM1     | 1.5 ± 0.1| 1.5 ± 0.1| 1.4 ± 0.1| 1.5 ± 0.1| 1.7 ± 0.1| 1.6 ± 0.2| 1.5 ± 0.2|
| GD1a    | 1.4 ± 0.2| 1.3 ± 0.2| 1.2 ± 0.2| 1.1 ± 0.2| 1.0 ± 0.2| 1.1 ± 0.2| 1.2 ± 0.1|

Raw macrophages were grown in the presence of 20 μCi of [14C]palmitate. Of the total radioactivity added, 924 ± 2 nCi (mean ± SEM; n = 7) was incorporated into lipid, no differences in the level of incorporation could be detected between the different treatments. Cells were treated for 24 h with varying concentrations of NB-DGJ (0–100 μM) or 50 μM Conduritol B epoxide (CBE). Radioactive lipids were extracted and quantitated. No consistent changes could be detected in the upper and lower bands of sphingolipids as they appear on TLC. Data are expressed as a percentage of total radioactive lipid (mean ± SEM; n = 4).

\* P < 0.05.

Fig. 4. Dose-dependent decrease in detergent-soluble and -insoluble GlcCer levels with NB-DGJ treatment in the presence and absence of CBE. RAW macrophages were grown in the presence of 20 μCi of [14C]palmitate for 48 h. Cells were treated for 24 h with 50 μM CBE and increasing concentrations of NB-DGJ (6–100 μM). TX-100-insoluble pellets were spun down and both the supernatant and pellet were extracted and radioactive lipids were quantitated. Results are mean ± SEM (n = 3). A: GlcCer levels in the presence of CBE and increasing NB-DGJ concentrations expressed as a percentage of each fraction. B: Sphingolipid levels in detergent-soluble and insoluble phases expressed as a percentage of total lipid ± NB-DGJ treatment. C: Detergent-insoluble sphingolipid levels ± NB-DGJ. D: GlcCer and GD1a levels with increasing NB-DGJ concentrations. Data are expressed as percentage of total radioactive GlcCer or GD1a recovered in each phase. GD1a insoluble, triangle; GD1a soluble, diamond; GlcCer insoluble, circle; GlcCer soluble, square.
LacCer sorting is a common property of GSL depleting agents, irrespective of their chemistry (Fig. 3C).

In order to study the changes in lipid composition that occur with NB-DGJ and CBE treatment, RAW cells were labelled with [14C]palmitate (Table 2). Labelling was performed for long periods as a surrogate for mass levels. Over a period of 48 h, labelling of phospholipids and sphingolipids was observed. GlcCer accounted for 1.2% of the total radioactivity. This was reduced by \( \frac{1}{2} \) to 0.6% after 24 h of NB-DGJ treatment. Apart from small changes in GD1a levels, no significant changes in the labelling of other phospholipids and sphingolipids could be detected. NB-DGJ concentrations had to be increased to 20 mM before appreciable cell death was observed (\( \sim \)20%).

**Detergent solubility of GSLs under conditions of GlcCer accumulation and depletion**

Previous work has suggested that increases of accumulated lipids in detergent-insoluble microdomains may play a role in sphingolipidoses (10, 23). Thus, we next tested the possibility that CBE or NB-DGJ treatment would lead to alterations in the detergent insolubility of GlcCer. For this comparison, we expressed the data as a percentage of GlcCer in each of the detergent-soluble or insoluble lipid pools. Whilst CBE treatment for 24 h caused GlcCer levels to double, no selective increase in detergent-insoluble GlcCer was observed (Fig. 4A). In addition, no increase in the detergent insolubility of BODIPY-LacCer could be detected on CBE treatment (results not shown). These results are unexpected in light of the recent speculation that the changes in trafficking in the SLSDs are due to increases in late endocytic or lysosomal microdomains (10). However, the lack of any increase in detergent insolubility of GlcCer may be the result of lysosomal GlcCer microdomains being a small percentage of the total GlcCer pool. Whether more sensitive methods of measurement would reveal any raft association following storage is beyond the scope of the present study. As expected, co-treatment with CBE and increasing concentrations of NB-DGJ caused progressive decreases in GlcCer levels (Fig. 4A). At high concentrations of NB-DGJ, even in the presence of CBE, GlcCer levels were lower than the controls with \( \sim \)50% and \( \sim \)40% decrease in the insoluble and soluble fractions, respectively, when compared to the control (Fig. 4A). We also investigated the partitioning of GlcCer in detergent soluble and insoluble fractions in the presence of NB-DGJ in the absence of CBE expressed as a percentage of total lipid. Greater that 90% of GlcCer was found in the detergent soluble fraction in both NB-DGJ treated and untreated cells (Fig. 4B). NB-DGJ treatment led to decreases in both the TX-100-soluble and insoluble GlcCer, however, there was a preferential decrease in the detergent-insoluble GlcCer (Fig. 4C, D). Levels of GlcCer were decreased by \( \sim \)60% in the detergent-insoluble pellet and \( \sim \)40% in the detergent-soluble phase. In contrast, GD1a levels also decreased but at higher (\( > \)12 \( \mu \)M) concentrations of NB-DGJ and the decreases were restricted to the TX-100-soluble phase (Fig. 4C, D). Thus, large decreases or increases in GlcCer led to changes in the trafficking of BODIPY-LacCer (Fig. 2, 3 and Table 2) but no consistent correlation could be made with the detergent insolubility of GSLs (i.e., GlcCer or GD1a) (Fig. 4), a measure of GSL microdomains (24, 25).

**Effect of changes in GlcCer on cholesterol distribution**

Since in SLSD fibroblasts accumulation of other GSLs has been shown to induce changes in cholesterol (7, 26), we were interested to determine whether pharmacological manipulation of GlcCer levels in fibroblasts could induce similar changes in cholesterol. The cholesterol binding antibiotic filipin was used to determine the subcellular distribution of cholesterol. We found that addition of CBE to inhibit GlcCer breakdown (Fig. 5A) or the addition of...
GlcCer levels. Although previous studies have shown that imino sugar treatment does not induce ceramide accumulation (27), we sought to address this issue by the addition of GlcSph, which has previously been shown to form intracellular GlcCer (21, 28). Since GlcSph and GalSph are relatively water-soluble, they are expected to rapidly enter the cell. Exogenous addition of these compounds led to the production of multinuclear cells (Fig. 6A), as previously reported (29, 30). At the concentrations used, these compounds caused a small amount of cell death (8% and 6% increase in propidium iodide staining for GlcSph and GalSph, respectively). However, coincubation of RAW cells with 25 μM NB-DGJ and 20 μM GlcSph for 48 h led to a restoration of BODIPY-LacCer targeting to the Golgi. This effect was specific since GalSph did not have the same effect and the punctate pattern of intracellular fluorescence persisted (Fig. 6A). GlcCer did not lead to large increases in cell death (3%) or restore altered trafficking. These results suggested that either GlcSph and/or its metabolism to GlcCer or higher GSLs is important for correct targeting of BODIPY-LacCer to the Golgi.

We sought to investigate whether exogenous GlcSph can lead to the formation of GlcCer in RAW macrophages. In these experiments, we labelled GSLs with [14C]galactose and treated with drugs as in A. After 2 days lipids were extracted and separated. [14C]GM3 was expressed as a percentage of the radioactivity in the phosphatidylcholine spot as an internal standard. Data are from two experiments.

Fig. 6. Exogenous GlcSph reverses NB-DGJ induced changes in BODIPY-LacCer sorting. RAW macrophages were grown on coverslips for 48 h in the presence of various combinations of 25 μM NB-DGJ, 20 μM GlcSph, 20 μM GalSph, or 40 μM GlcCer. A: Distribution of BODIPY-LacCer. B: Exogenous GlcSph led to the production of GM3. Cells were grown in the presence of 10-[14C]galactose and treated with drugs as in A. After 2 days lipids were extracted and separated. [14C]GM3 was expressed as a percentage of the radioactivity in the phosphatidylcholine spot as an internal standard. Data are from two experiments.

Exogenous glucosylsphingosine restores altered trafficking

Since the addition of NB-DGJ leads to an inhibition of ceramide utilisation by ceramide glucosyltransferase as well as the inhibition of GlcCer synthesis, the effects on altered trafficking could be a result of the increase in ceramide concentration as opposed to the decrease in GlcCer directly to the culture medium (not shown) can induce a punctate endocytic cholesterol distribution in fibroblasts (Fig. 5A). We also repeated these experiments in RAW macrophages. In these cells, cholesterol was concentrated within the plasma membrane as well as intracellularly with some staining in a perinuclear area (Fig. 5B). In macrophages, treatment with CBE, NB-DGJ, or GlcCer for 24 h induced slight increases in punctate staining but the results were equivocal (data not shown). However, at 48 h these treatments induced a more punctate pattern of filipin staining (Fig. 5B), reminiscent of that seen in fibroblasts. Control RAW cells have cholesterol levels of approximately 37 ng/μg protein (Fig. 5C). Short-term treatment of RAW cells with CBE, GlcCer, and NB-DGJ (4 h) cause only subtle changes in cholesterol mass levels. However, longer term treatment (48 h) with all three compounds resulted in significant increases in cholesterol with the greatest effects observed with exogenously added GlcCer (71 ± 12 ng/μg). Thus, both increases and decreases in GlcCer levels induce changes in cholesterol levels and/or its intracellular distribution.

The present results show that both depletion or accumulation of GlcCer lead to a change in cholesterol levels and distribution in macrophages and fibroblasts. We have not identified the mechanism(s) by which alterations in GlcCer may elevate intracellular cholesterol. Two possibilities are that alterations in endosomal/lysosomal lipid composition cause cholesterol to become trapped in endocytic structures (9) or that GlcCer is specifically required for a critical step in cholesterol transport (e.g., to the ER).

Exogenous glucosylsphingosine restores altered trafficking

Since the addition of NB-DGJ leads to an inhibition of ceramide utilisation by ceramide glucosyltransferase as well as the inhibition of GlcCer synthesis, the effects on altered trafficking could be a result of the increase in ceramide concentration as opposed to the decrease in GlcCer.
It is notable that GlcCer has also been implicated as being important in the transport of tyrosinase from the Golgi to the melanosome in melanocytes (21), suggesting that GlcCer has specific roles in other vesicular pathways. Whether GlcCer’s function in protein sorting is always restricted to its participation in microdomains and whether GlcCer function is dependent on its topology are subjects for future investigation. A correlation between altered trafficking of BODIPY-LacCer and the level of GlcCer, but not other GSLs, within the cell was observed in CBE treated macrophages. From previous studies, NB-DGJ enters cells rapidly (within minutes), and at a concentration of 50 μM inhibits GlcCer transferase by over 90% of de novo GlcCer synthesis (11). In this study, treatment with NB-DGJ led to a ~50% depletion of total GlcCer in prelabelled cells. This may be explained by the fact that GlcCer is unusual amongst the GSLs in that synthesis is on the cytoplasmic leaflet of the early Golgi and has to be translocated across the Golgi membrane for the synthesis of higher GSLs. This implies that there are two pools of GlcCer in cells topologically separated in the luminal and cytoplasmic leaflet of the Golgi and perhaps also in other organelles in the secretory and endocytic pathway (31). Since turnover of each pool may occur in different subcellular locations and by different enzymes it may be expected that each pool has different turnover rates (31).

The effects of GlcCer depletion on lipid endocytic trafficking suggest that extensive inhibition of GlcCer synthesis in substrate reduction therapy may be undesirable since GlcCer depletion leads to a similar targeting of BODIPY-LacCer to that observed in the SLSDs. However, these effects were observed at at least 5-fold higher concentrations of imino sugars than those achieved therapeutically in Gaucher patients (3) where efficacy of substrate reduction therapy was demonstrated. It may be expected that each pool has different turnover rates (31).

It will be interesting to determine whether there are any adaptive responses made by the cell if long term depletion is sustained. More potent compounds that achieve higher degrees of GSL depletion would be anticipated to be more likely to cause local or systemic changes in endocytic sorting than amphiphilic imino sugars. Establishing what the functional consequences of altered endocytic sorting are on cell function may lead to greater insight into the cellular pathology of GSL storage diseases.18

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