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Glycosphingolipid Mediated Caveolin-1 Oligomerization

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Abstract

We have previously demonstrated an association between the accumulation of the glycosphingolipid globotriaosylceramide (Gb3) and the loss of high molecular weight oligomers in the aortas of α -galactosidase A-knockout mice, a model of Fabry disease. In the present study the molecular basis for the association between glycosphingolipids and caveolin-1 oligomerization was further investigated. Cellular glycosphingolipids were selectively depleted by treatment with a series of sphingolipid synthesis inhibitors, including D-*threo*-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol, fumonisin B1 and myriocin. The depletion of glycosphingolipids resulted in the loss of high molecular mass oligomers of caveolin-1 in plasma membranes of cultured ECV-304 cells as well as in the caveolar fractions of Hela cells as measured by immunoblotting. The disruption of caveolin-1 high molecular weight oligomer formation caused by changes of composition of glycosphingolipids may be directly involved in the interruption of cellular functions including caveolar stabilization, membrane trafficking and signal transduction. These results suggest a specific role for glycosphingolipids in the caveolar co-localization and oligomerization of caveolin-1.

Keywords

Glycosphingolipids; Caveolin-1; Lipid raft; Fabry disease; Globotriaosylceramide; Glucosylceramide synthase

Introduction

Considerable attention has been focused on the biology of lipid rafts generally and caveolae specifically as important sites of cell signaling. Caveolae are small, uncoated pits in the plasma membrane [1]. They are a characteristic feature of endothelial cells. Caveolae are defined by the presence of the eponymously termed protein caveolin-1. Caveolae are considered to be a form of lipid raft. Caveolin-1 is clearly critical for caveola formation since the expression of caveolin-1 in cells normally lacking the protein results in the formation of caveolae. Similarly, the experimental loss of caveolin-1 results in the loss of caveolae. Approximately 100 to 200 caveolin molecules are present in each caveola, most in an oligomeric form [2]. Caveolin-1 not only plays a critical role in the structure of lipid rafts and its role as scaffold for the assembly of signaling molecules. Among such molecules are endothelial nitric oxide synthase (eNOS) [3] and c-Src [4]. Considerably less is known regarding the role of specific lipids in the function and structure of caveolae, despite the fact

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that caveolae are highly enriched in both cholesterol and sphingolipids. Caveolin-1 is insoluble in nonionic detergents such as Triton X-100. Triton X-100 membrane fractions are also enriched in cholesterol and sphingolipids [5,6]. A number of studies have suggested that the transmembrane domain of caveolin-1 and these lipids are of sufficiently close proximity to interact with one another [4]. Lysosomal storage diseases, specifically the sphingolipidoses, provide potentially important models for studying the role of lipids in caveolar function. One such example is Fabry disease, the result of a deficiency in α -GlcA with the accumulation of α -1-4 linked GSLs, most notably Gb3. Gb3 accumulation is not restricted to the lysosome but is also markedly increased in the plasma membranes and caveolae of endothelial cells [7]. We have previously investigated the endothelial dysfunction in the α -GlcA null mouse, a model of Fabry disease [8–10]. Three vascular abnormalities are readily observed including sensitivity to oxidant induced thrombosis [11], accelerated atherogenesis [8] and impaired relaxation in aortic rings [10]. Each of these abnormalities may be the result of decreased NO bioavailability resulting from eNOS uncoupling. In support of this mechanism, we observed that aortic endothelial cells from α -GlcA knockout mice are characterized by lower eNOS levels and activity [12]. In that report we observed that Gb3 accumulation was associated with the loss of high molecular weight oligomers of caveolin-1. In this report we have studied the further role of glycosphingolipids in caveolin-1 oligomerization using *in vitro* models of glycosphingolipid depletion and selective inhibitors of sphingolipid synthesis.

Materials and Methods

Materials

Et-DOP4 was synthesized as previously described [13]. Myriocin and fumonisin B1 were purchased from Sigma-Aldrich (Atlanta, GA).

Cell treatments

Human ECV304 and Hela cells were grown in Opti-MEM/EMEM medium (2:1) supplemented with 5% FBS, 4.5 g/liter D-glucose, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. After recovery from Trypsin-EDTA treatment (48 h), cells were exposed to inhibitors including D-*threo*-ethylenedioxyphenyl-2-palmitoylamino-3-pyrrolidino-propanol (Et-DOP4), fumonisin B1 (FB1), or myriocin in serum-free Opti-MEM/EMEM for 48 h at the concentrations indicated. Stock solutions of EtDO-P4, FB1 and myriocin, were prepared in 100% Me₂SO and further diluted with serum-free medium prior to use.

Caveolar isolation

Caveolar membranes, highly enriched in caveolin-1, were isolated from cultured Hela cells either treated with Et-DOP4 or without treatment by using a non-detergent method, taking advantage of the unique buoyant density of caveolar membranes as described previously [14]. The caveolar fractions from the first OptiPrep gradient (20% to 10%) were collected. The immunoreactivity of caveolin-1 in each caveolar fraction (0.35 ml) of cultured Hela cells was analyzed using an anti-human caveolin-1 polyclonal antibody under non-denaturing conditions.

Lipid analyses

Total cellular lipids were extracted from both treated and untreated human ECV304 cells as previously reported [7]. After partitioning by the addition of chloroform/methanol/0.9% NaCl at a final ratio of 2/1/0.8 to form two phases, the neutral glycosphingolipids in lower chloroform/methanol phase were washed with methanol and 0.9% NaCl and purified by

base- and acid-treatments [15]. All upper liquid phases containing gangliosides, collected from first chloroform/methanol/0.9% NaCl partition and following methanol/0.9% NaCl washings of lower organic phase, were passed through a Sep-PaK C18 column (Waters, Milford, MA) to purify gangliosides. The C18 column was prewashed with solvents in the following sequence: 5 ml of methanol, 10 ml of chloroform/methanol (1:1), 5 ml of methanol and 8.5 ml of methanol/0.9% NaCl (1:0.7). The upper-layer lipid samples were reappplied into C18 column twice and washed with 10 ml of water to remove the salts. Cell gangliosides were eluted from the column with 10 ml of chloroform/methanol (1:1) and dried under a stream of nitrogen gas. The depletion of glucosylceramide, lactosylceramide, Gb3, cholesterol and sphingomyelin in treated and untreated ECV304 cells were analyzed by high performance thin layer chromatography as previously detailed [15]. The levels of gangliosides were determined by a one-step separation in a solvent system consisting of chloroform/methanol/0.2% CaCl₂·2H₂O (55/45/10).

Immunoblot analysis

Cell lysates was first analyzed for protein content with bicinchoninic acid/copper sulfate solution (50:1) using bovine serum albumin as a protein standard. Equal amounts of proteins from total cellular lysates of ECV304 cells or equal volume of caveolar fractions from cultured Hela cells were subjected to electrophoretic separation by a 6–12% gradient SDS-PAGE without preheating and β -mercaptoethanol reduction [15]. The transferred nylon membranes were blocked in Tris-buffered saline (pH 7.6) containing 5% skimmed dry milk and 5% calf serum overnight and probed with rabbit polyclonal antibodies (Abcam, Cambridge, MA) against mouse caveolin-1 and CD151. The primary antibodies were detected with goat anti-rabbit IgG and visualized with the ECL-plus system.

Statistical analysis

The data are expressed as the mean $\pm$ S.E. Statistical analyses were performed with either the student's *t*-test or by two-way ANOVA and the differences between means were considered to be significant for a P value < 0.05.

Results

Primary cultures of aortic endothelial cells derived from Gla knockout mice are characterized by an excess Gb3 accumulation and the concomitant loss of caveolin-1 high molecular mass oligomers [7,12]. To better understand the general role of glycosphingolipids in the oligomerization of caveolin-1, a pharmacological strategy for altering GSL content in cell membranes was employed. ECV304 cells were treated with the small molecule inhibitors Et-DOP4, fumonisins B1 (FB1) and myriocin to block glucosylceramide synthase, ceramide synthase and serine palmitoyl-transferase respectively. Et-DOP4, an active GlcCer synthase inhibitor with nanomolar inhibitory activity, depletes all glucosylceramide based GSLs [13]. Myriocin inhibits the synthesis of long chain bases and in turn both ceramide and dihydroceramide and therefore blocks the de novo formation of all sphingolipids including sphingomyelin and glycosphingolipids [16]. FB1 inhibits the acylation of both dihydrosphingosine and sphingosine and thus blocks the de novo synthesis of all sphingolipids with the exception of sphingosine-1-phosphate [17].

ECV304 cells were treated with GSL inhibitors or vehicle and the crude cellular lipids were extracted, partitioned into neutral GSLs and gangliosides and separated by thin layer chromatography (Figure 1A). In the presence of Et-DOP4 for 48 h the neutral GSLs, including glucosylceramide, lactosylceramide and Gb3 levels were 90 percent lower than those in vehicle treated cells. Sphingomyelin levels, however, were increased (Figure 1B). FB1 and myriocin treatment resulted in a similar pattern in the decrement in neutral GSLs.

The cellular gangliosides were purified from methanol/0.9% NaCl phases and separated with a solvent system consisting of chloroform/methanol/0.2% CaCl_2 , (55/45/10, v/v/v). A representative thin layer chromatogram is shown in Figure 2A comparing the acidic glycolipids with known standards. An unknown dense band migrated above sphingosine which did not change following exposure to any of the inhibitors. The levels of gangliosides GM1, GM2, GM3 and GD1a in cultured ECV304 cells were highly sensitive to Et-DOP4 treatment but less sensitive to the other inhibitors (Figure 2B). FB1 treatment decreased gangliosides GM1 and GM2 by more than 85 percent, but was less active in lowering gangliosides GM3 and GD1a. Myriocin had no observed effect in lowering ganglioside GM1, but modestly lowered gangliosides GM2, GM3 and GD1a. The differences in ganglioside levels between FB1 and myriocin may reflect the different sites of action of these inhibitors. Myriocin blocks de novo long chain base synthesis. FB1 inhibits the acylation of both sphingosine and dihydrosphingosine and thus may inhibit glycolipid synthesis occurring through de novo synthetic routes as well as through recycling pathways. ECV304 cell cholesterol levels following treatment with Et-DOP4, FB1 and myriocin were also analyzed by high performance thin layer chromatography. Exposure to the three inhibitors resulted in a similar change in cholesterol content. The reduction of cholesterol was approximately 20 percent following a 48 h exposure to each inhibitor (Figure 2C). The levels of high oligomer caveolin-1 were measured by immunoblotting following treatment with the sphingolipid synthesis inhibitors. The levels of high molecular weight oligomers of caveolin-1 were significantly lower in the presence of each inhibitor. This was most notable for those oligomers with molecular weights in excess of 400 kDa. A representative Western blot of caveolin-1 in total cell lysates of ECV304 cells is shown in Figure 3A. All treatments significantly lowered the caveolin-1 oligomers at molecular mass higher than 400 kDa. Et-DOP4 treatment at 0.2 μM for 48 h lowered the top very high caveolin-1 oligomers, but had less effect on the 250 kDa oligomers when compared to FB1 and myriocin (Figure 3B). Higher concentrations of myriocin and FB1 were associated with comparable decrements in the 400 kDa oligomers but failed to demonstrate a concentration dependent reduction in the 250 kDa oligomers. HeLa cells were studied to evaluate the generalizability of the observed changes in caveolin-1 and to confirm that the changes were due to loss of high molecular weight oligomers within caveolae as opposed to a mistrafficking of caveolin-1. HeLa cells were exposed to Et-DOP4 for 48 hours at a concentration of 0.25 μM . Caveolar fractions were collected using Opti-Prep, a non-detergent method and caveolin-1 in caveolar fractions was detected by immunoblotting. High molecular mass oligomers (> 400 kDa) of caveolin-1 were not detected in the presence of the glucosylceramide synthase inhibitor (Figure 4). Levels of both 250 kDa oligomers and of caveolin-1 monomers were not detectably different compared to vehicle treated control cells.

Discussion

This study extends recent work attempting to better understand the role of GSLs in the regulation of those caveolar associated cell signaling events that may provide insights into the basis for the vasculopathy associated with Fabry disease. It has been previously reported in both clinical studies and animal models of Gla deficiency, that abnormalities in vascular reactivity, enhanced thrombosis and accelerated atherogenesis may be tied to the uncoupling of eNOS [10,18]. In the Gla knockout mouse, the endothelial cell defect is marked by the accumulation of Gb3 within caveolae and a secondary decrease in cholesterol content [7]. This results in both lower eNOS expression and the uncoupling of eNOS with its conversion to a superoxide generating enzyme. Although decreased NO bioavailability through eNOS dysregulation can arise from several potential mechanisms, the post-translational regulation of eNOS that is dependent on the direct binding to caveolin-1 and dissociation from caveolae following cell signaling has been a particular focus of inquiry. The association of eNOS with the plasma membrane through palmitoylation and myristoylation sites appears to

depend on the lipid composition of the caveolae [19,20]. However, the importance of caveolar lipids in the regulation of eNOS activity is based almost exclusively on studies restricted to evaluating the role of cholesterol in caveolae. Cholesterol depletion with cyclodextrin or HDL or cholesterol displacement with oxidized LDL lowers eNOS activity by redistribution of eNOS from caveolae [21]. However, the role of other caveolar lipid components in eNOS redistribution, most notably sphingomyelin and glycosphingolipids, has not been studied. Caveolae are small, uncoated pits in the plasma membrane. They are an abundant feature of endothelial cells. Caveolae are defined by the presence of caveolins. They are considered to be a form of lipid raft. Cholesterol and sphingolipids are critical components of lipid rafts and at least the former lipid is believed to be critical for caveola formation [22]. Caveolin-1 is clearly critical for caveola formation since the expression of caveolin-1 in cells normally lacking the protein results in the formation of caveolae. Similarly, the experimental loss of caveolin-1 results in the loss of caveolae. Approximately 100 to 200 caveolin molecules are present in each caveola, most in an oligomeric form [2]. The role of lipids in general and of gangliosides in particular in the co-regulation of c-Src and caveolin-1 has been more extensively studied. Caveolin-1 interacts with c-Src within a lipid raft domain that is enriched in glycosphingolipids. The manipulation of the lipid composition of these domains markedly affects this interaction. Conversely, Src regulated caveolin-1 function through the phosphorylation of caveolin-1 [23]. Recently, Prinetti and coworkers reported that ganglioside GM3/caveolin-1 interactions are potentially important in the motility of ovarian carcinoma cells [24]. Although a correlation between increased ganglioside GM3 levels and increased caveolin-1 expression was reported, the role of GM3 in caveolin-1 oligomerization was not studied. Caveolins are synthesized on the rough endoplasmic reticulum and transit through the Golgi complex prior to trafficking to the plasma membrane. At some point during this synthetic route, caveolins change from a monomeric form to an oligomeric form. Studies utilizing a caveolin-1-GFP fusion construct are consistent with the formation of the caveolae in the Golgi complex [25]. Cholesterol appears to be important for this process [26]. The addition of cholesterol appears to decrease the transit time through the Golgi complex. Cholesterol binds directly to caveolin-1 as demonstrated by photo-active crosslinking studies, plasma membrane cholesterol enrichment in cells expressing caveolin-1 and numerous structural studies defining cholesterol binding motifs [22]. The disruption of caveolin-1 oligomerization should be viewed as distinct from the question of whether glycosphingolipids themselves are important regulators of protein sorting to caveolae. Previously, we reported that glycosphingolipid depletion did not alter the sorting of annexin-II and caveolin-1 in NIH-3T3 cells [14]. Subsequently, the absence of changes in total lipid raft levels of several proteins, including phospholipase C β 1, phospholipase C δ 1, phospholipase C γ 1, PDGF receptor, annexin II, c-Raf-1 and RAS, were observed to be unchanged with glucosylceramide synthase inhibition [27]. In the present study we evaluated the effects of GSL depletion on caveolin-1 expression and oligomerization. Using three independent agents to lower cellular GSLs, a marked decrement in high molecular weight caveolin-1 oligomers was observed. A corresponding albeit smaller decrement in cholesterol was noted. The basis for the reproducible decrement in cholesterol content was not apparent. The three specific inhibitors employed are individually active at distinct sites in the anabolic pathways for sphingolipids. The equivalence in the response of each to the decrease in the very high molecular weight caveolin-1 oligomers suggests that the effects were likely mediated through one or more GSLs as opposed to a non-glycosylated sphingolipid including sphingosine, ceramide or sphingomyelin. Coupled with prior observations on the role of Gb3 in caveolar structure in aortic endothelial cells, these data are consistent with an important functional role for GSLs in the establishment of higher order caveolar structure. One caveat is worth considering in interpreting these findings. Caveolin-1 easily forms aggregates and thus the observed changes in the ratio of high to low molecular weight oligomers should be interpreted cautiously. However, because the only difference between the EtDO-P4 treated and

untreated cells was the exposure to the glucosylceramide synthase inhibitor, the observed difference would appear to be due to the changes in cellular glycosphingolipids and not experimental artifact. More direct proof of this difference would be a formal examination of the ultrastructural changes in the caveolae in the treated and untreated cells. Unfortunately, the ECV-304 cell line does not lend itself to these studies and therefore confirmation of these findings will require the use of an alternative *in vitro* model. One unanswered several unanswered questions in this study is whether the loss of high molecular weight oligomers is due to a change in caveolar GSL, cholesterol, or some combination of the two. A direct association between caveolin-1 and a specific GSL based on either its glycan or ceramide structure is intriguing to consider for several possibilities. A direct interaction between caveolin-1 molecules and GSLs may be important for the creation or maintenance of the structure and integrity of acaveola. Prior work has demonstrated that in the presence of EtDO-P4, protein sorting to lipid rafts occurs normally [14]. This suggests that the effect of depleting glucosylceramide-based GSLs on caveolin-1 levels and oligomerization may not be part of a generalized protein sorting defect. However, caveolar membranes are assembled at the distal Golgi apparatus, the site of higher order GSL formation. Thus the co-assembly of caveolin-1 oligomers and GSLs may be a necessary step in the establishment of the caveolar structure.

The role of GSLs in sorting and trafficking of caveolar membranes will be important to discern. If GSLs are central to this process, then there may be an association between cell specific expression of specific GSLs and the presence of caveolae. Because there are more than 300 distinct mammalian GSLs based only on their glycans and not specific lipid composition, a definitive understanding of GSL/caveolin-1 interactions will require significant future work. However, the relationship between cellular caveolae and the expression of specific GSLs may help to focus such studies. For example, caveolae are most notably present in vascular endothelial cells as is Gb3. Finally, discerning the roles of GSLs and caveolar structure and function holds implications for the understanding and treatment of disorders of GSL metabolism, such as Fabry disease. If aberrant levels of Gb3 result in altered caveolar function and uncoupling of eNOS, then any effective therapy in reversing this abnormality, such as enzyme replacement with recombinant Gla, ought to be associated with the recovery of normal caveolar structure. Because Fabry disease is rare and the important complications such as stroke and myocardial infarction are infrequent, the time to outcome for the assessment of therapies is inordinately long. Therefore the identification of appropriate biomarkers for disease such as loss of caveolin-1 oligomerization is potentially useful for assessing therapies and predicting disease outcomes.

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Abbreviations

eNOS	Endothelial Nitric Oxide Synthase
EtDOP4	D- <i>threo</i> -Ethylenedioxyphenyl-2-Palmitoylamino-3-Pyrrolidino-Propanol
FB1	Fumonisin B1
Gla	α -Galactosidase A
Gb3	Globotriaosylceramide

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Figure 1. Depletion of GSLs from cultured ECV304 cells with sphingolipids synthesis inhibitors
Cells were treated in serum-free medium for 48 h. GSLs were extracted, purified and analyzed by high performance thin layer chromatography as described in the Methods section. A. A representative thin layer chromatography (TLC) plate of ECV304 cells treated with Et-DOP4, FB1 and myriocin at the indicated concentrations.

Abbreviations: GlcCer: Glucosylceramide; LacCer: Lactosylceramide; Gb3: Globotriaosylceramide; SM: Sphingomyelin B. The data represent the mean $\pm$ SEM from 3 experiments. * denotes $p < 0.05$ calculated by two-way ANOVA of GraphPad Prism ($n = 3$).

Figure 2. Depletion of gangliosides from cultured ECV304 cells with sphingolipids synthesis inhibitors

The lipid sample preparation and thin layer chromatographic analysis were detailed in the Methods section. A. A representative high performance thin layer chromatography plate displaying the ganglioside pattern of cultured ECV304 cells after a 48-h treatment with inhibitors. B. Comparison of ganglioside levels in untreated and treated ECV304 cells with sphingolipid inhibitors. The data represent the mean $\pm$ SEM from 3 separate experiments. * denotes $p < 0.05$ as determined by two-way ANOVA using GraphPad Prism. C. The reduction of cholesterol content in inhibitor treated ECV304 cells. Crude cellular lipids were normalized to 20 nmol phospholipid phosphate prior to separation by high performance thin layer chromatography using a solvent system consisting of chloroform/acetic acid (9:1, v/v). The plates were scanned and analyzed ImageJ software, and the levels of cholesterol were quantified by comparison to untreated control samples.

Figure 3. The depletion of GSLs induces deoligomerization of caveolin-1 in cultured ECV304 cells

Total cellular proteins were lysed with 1% Triton X-100, quantified using BCA assay, separated electrophoretically in a 6- 12% gradient SDS-PAGE, and analyzed by immunoblotting in non-reducing and non-heating conditions. A. The Western blot is representative of three independent experiments that yielded equivalent results. B. the densitometric data summarized from the immunoblots of the individual experiments.

Figure 4. GSL depletion eliminates high molecular weight oligomers of caveolin-1 in caveolar fractions of cultured HeLa cells

Equal amounts of the initial Opti-prep gradient fractions were loaded into a 6–12% gradient SDS-PAGE. Caveolar caveolin-1 was verified by immunoblots with a polyclonal antibody against human caveolin-1 and compared to immunoblots using monoclonal antibody against human CD151 as a control.