Caveolin-associated Accumulation of Globotriaosylceramide in the Vascular Endothelium of α-Galactosidase A Null Mice*

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Cardiovascular complications, including stroke and myocardial infarction, result in premature mortality in patients with Fabry disease, an X-linked deficiency of α-galactosidase A (α-Gal A). The enzymatic defect results in the deposition of globotriaosylceramide (Gb3) in the vascular endothelium. To better understand the underlying pathogenesis of Fabry disease, the caveolar lipid content of primary cultured mouse aortic endothelial cells isolated from α-Gal A null mice was measured. Lipid mass analysis revealed that the excessive Gb3 in cultured α-Gal A-deficient mouse aortic endothelial cells accumulated in endothelial plasma membrane caveolar fractions. The levels of glucosylceramide and lactosylceramide increased in parallel with Gb3 levels in an age-dependent manner, whereas globotetraosylceramide (Gb4) levels reached maximal levels by 6 months of age and then rapidly decreased at older ages. The levels of cholesterol enriched in caveolar membranes declined in parallel with the progressive deposition of Gb3. Depleting Gb3 with recombinant human α-Gal A protein or d-threo-ethylenedioxyphenyl-P4, an inhibitor of glucosylceramide synthase, restored cholesterol in cultured α-Gal A-deficient mouse aortic endothelial cell caveolae. By contrast, recombinant human α-Gal A was less effective in normalizing the cholesterol content. These results demonstrate the caveolar accumulation of glycosphingolipids in an in vitro model of a lysosomal storage disease and raise the possibility that dynamic changes in the composition of plasma membrane lipid microdomains may mediate the endothelial dysfunction seen in Fabry disease.

Fabry disease is one of 42 recognized lysosomal storage disorders. Fabry disease results from the absence or decreased activity of the lysosomal hydrolase α-galactosidase A (α-Gal A). α-Gal A is transmitted as an X-linked recessive gene (1, 2). The lack of enzymatic activity of α-Gal A in Fabry patients leads to the progressive accumulation of glycolipids with α-galactosyl linkages, primarily globotriaosylceramide (Gb3). Gb3 accumulates in the lysosomes of numerous tissues throughout the body, most notably the vascular endothelium. Fabry disease is a systemic disorder and functionally affects the skin, eyes, kidney, heart, and autonomic nervous system. The premature mortality of affected patients, however, primarily occurs from cardiovascular catastrophes such as strokes and myocardial infarctions (3). Although it is well documented that cardiovascular abnormalities are the most common and predominant clinical manifestation of Fabry disease, the precise biological role of Gb3 deposition in the pathogenesis of the large vessel vasculopathy in Fabry disease remains unknown.

The generation of knock-out mice lacking α-Gal A activity has provided a useful model for the study of the vasculopathy in Fabry disease (4). Although these mice do not display a spontaneous vasculopathy, they exhibit a robust thrombotic response to oxidant-induced injury (5) and accelerated atherosclerosis when bred on an apo-E1 null background (6). In addition, these mice display impaired contraction to phenylephrine and impaired acetylcholine-stimulated vasorelaxation in their aortic rings (7). All three findings are age-dependent. Because these phenotypes are observed in either living mice or isolated vessels, establishing a mechanistic basis for the vasculopathy has been difficult. As an initial step in studying potential mechanisms, a method for growing primary cultures of aortic endothelial cells derived from the α-Gal A null mice was established (7). These cells retain high levels of Gb3 and thus provide a potentially useful reagent for probing the Fabry phenotype.

The vascular models established to date in the α-Gal A null mice are consistent with the presence of an endothelial signaling defect in nitric oxide generation. How an enzyme deficiency resulting in the lysosomal accumulation of Gb3 might result in the impairment in agonist-stimulated nitric oxide formation is unknown. Plasma membrane-associated glycosphingolipids have long been demonstrated to regulate the activity of signaling molecules, including receptor tyrosine kinases (8), Src kinases (9), and phospholipase C (10). Receptor-mediated endocytosis is regulated by glycosphingolipids as well. However, little if any data exist demonstrating a cross-talk between lysosomes and plasma membrane signaling. Furthermore, with the exception of Niemann-Pick C disease, little data exist demonstrating the accumulation of sphingolipids outside of the lysosome in storage diseases.

To evaluate whether the glycosphingolipid content in plasma membrane caveolar fractions is affected in the setting of lysosomal storage disease, aortic endothelial cells from α-Gal A null mice were studied. We report that absence of α-Gal A activity

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2 The abbreviations used are: α-Ga A, α-galactosidase A; Gb3, globotriaosylceramide; MAEC, mouse aortic endothelial cell; d-threo-ethylenedioxyphenyl-P4, d-threo-ethylenedioxyphenyl-2-palmitoylaminolmo-3-pyrrolidinopropanol; FBS, fetal bovine serum; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GlcCer, glucosylceramide; LacCer, lactosylceramide; HPTLC, high performance thin layer chromatography.

3 J. Park and J. A. Shayman, unpublished data.
in mouse aortic endothelial cells (MAECs) results in the age-dependent accumulation of globo series glycosphingolipids in plasma membrane caveolin-associated lipid rafts.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant human α-Gal A enzyme (Fabrazyme®), produced in a Chinese hamster ovary mammalian cell expression system, was a kind gift from Genzyme Corp. (Cambridge, MA). d-threo-Ethyleneoxyphenyl-2-palmitoylamino-3-pyrrolidinopropanol (d-t-EtDO-P4) was synthesized in our laboratory as previously described (11). Endothelial cell growth supplement was purchased from BD Biosciences. Fetal bovine serum (FBS) was obtained from America Type Culture Collection (ATCC) (Manassas, VA). Collagen type I, collagenase type I, and Percoll were from Sigma-Aldrich. OptiPrep was purchased from Accurate Chemical & Scientific Corp. (Westburg, NY). All of the lipid internal standards were acquired from Matrex ( Pleasant Gap, PA).

**Animals**—C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). α-Gal A knock-out mice with a 129SV/JxC57BL/6 cross-breeding background were bred and maintained under standard protocols at the University of Michigan as previously described. The genotype of α-Gal A-deficient mice was confirmed by PCR analysis. The care and euthanasia of the mice was in accordance with the standards in “The Guide for the Care and Use of Laboratory Animals” DHEW publication number (National Institutes of Health) 86-23, revised 1985.

**Cell Cultures**—The primary MAECs were isolated from α-Gal A knock-out and wild-type C57BL/6J mice, at ages from 1 to 8 months, by utilizing a non-mechanical and non-enzymatic method with minor modifications as described previously (7). Penicillin, streptomycin, and fungizone significantly delayed endothelial cells growth from explanted aortic rings and were therefore omitted from RPMI plating medium. The outgrowth of endothelial cells from aortic rings was markedly facilitated within first 72 h in the absence of antibiotics. Aortic rings thus were discarded at culture day 3 to avoid the possible contamination of non-endothelial cell types (12). After removing the aortic rings, cells were maintained in completed RPMI medium consisting of 20% FBS, 2 mM L-glutamine, 1 × nonessential amino acid, 0.05 mg/ml endothelial cell growth supplement, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.1 mg/ml heparin until confluent.

**Cell Treatments**—Because high concentrations of serum (≥15%) in culture medium induced increased Gb3, the percentage of serum in medium was gradually reduced after cells were serially passaged. MAECs at passages 0 and 1 (P0 and P1) were grown in RPMI medium containing 20% FBS, and MAECs at P2 and P3 were cultured in 15% FBS–RPMI medium. MAECs at P4, only maintained in 10% FBS–RPMI medium in excess of 2 days, were used for experiments unless otherwise indicated. A stock solution of d-t-EtDO-P4 dissolved in 100% Me2SO was diluted with plain RPMI, and recombinant human α-Gal A was freshly reconstituted with plain RPMI medium just before use. Treated MAECs, maintained in 10% FBS–RPMI, were exposed to either 0.5 µM d-t-EtDO-P4 or 10 µg/ml recombinant human α-Gal A for 1 or 2 days before being harvested.

**Isolation of Caveolae**—Caveolae were isolated from cultured MAECs using a detergent-free method that takes advantage of the unique buoyant density of caveolar membranes (13). Briefly, cells grown in 150- × 25-mm dishes were collected by scraping in buffer A (0.25 M sucrose/20 mM Tricine/1 mM EDTA, pH 7.8). Whole cells were fractionated by homogenization, and plasma membrane fractions were separated by centrifugation in buffer A containing 30% Percoll. Caveolar membranes were further purified from plasma membrane fractions by centrifugation in a 20% to 10% OptiPrep linear gradient and concentrated by a second centrifugation in a 5% OptiPrep-Buffer A discontinuous gradient. A distinct opaque band of caveolar membrane in the 5% OptiPrep was collected for lipid extraction and mass analysis.

**Lipid Analysis**—The total protein concentration of each sample was determined by the bicinchoninic acid assay (Sigma) with bovine serum albumin as a standard. Equal amounts of fraction proteins were mixed with sample loading buffer consisting of 50 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 2% SDS (w/v), and 0.003% bromphenol blue (w/v). Samples separated under non-reducing conditions were directly subjected to SDS-PAGE using a 6–13% gradient, and samples separated under reducing conditions were heated for 5 min at 90 °C in sample loading buffer containing 1.5% 2-mercaptoethanol before electrophoresis. 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 2. The primary antibodies were detected with goat anti-rabbit IgG and visualized with the ECL-plus system. Annexin II was detected with a mouse monoclonal antibody (BD Transduction Laboratories, San Jose, CA).

**Lipid Extraction**—The extraction of total cellular lipids followed a protocol as previously described by Shu et al. (14). The caveolar membrane lipids were extracted from caveolar fractions with chloroform and methanol. A mono-phase was formed by the addition of chloroform and methanol to the caveolar membranes at a volume ratio of 1:2:0.8 (chloroform/methanol/caveolar fraction). After sonication in a bath sonicator for 15 min, caveolar proteins were precipitated from the mono-phase mixture by centrifugation at 2500 × g for 30 min. The supernatant was collected with a Pasteur pipette into a new glass tube, and organic and water dual phases were created by the addition of 4 ml of chloroform, 3 ml of methanol, and 4.2 ml of 0.9% NaCl solution. The sample was centrifuged at 1250 × g for 5 min. The upper aqueous phase was discarded. The lower organic phase was transferred into another new glass tube and extensively washed with chloroform, methanol, and 0.9% NaCl, at the theoretical ratio of 1:1:1 (v/v), four times to remove co-extracted OptiPrep in caveolar lipids. The lipids in chloroform portion were dried under a stream of nitrogen and redissolved into chloroform/methanol (2:1, v/v).

**Lipid Analysis**—Neutral glycosphingolipids were purified from crude whole cellular and caveolar membrane lipids by base and acid hydrolysis as described in detail (7). Briefly, samples were normalized for equal amounts of lipids using a total phospholipid assay with potassium phosphate monobasic as a standard (15). A portion of the lipids (100 nmol of total phos-
phospholipid phosphate) was incubated with NaOH (0.21 M) in methanol at 37 °C for 1 h. After neutralization with HCl (0.25 M), the lipids in the chloroform phase were incubated with 0.05 M HCl/25 mM HgCl₂ at 37 °C for 15 min. The extracted neutral lipids were then purified with methanol/30 mM EDTA (2:1.6, v/v) once and methanol/water (2:1.6, v/v) twice. The neutral glycosphingolipids Gb₃, Gb₄, GlcCer, and LacCer were analyzed with two successive solvent systems as previously reported (7). Alternatively, GlcCer and LacCer were separated in a solvent system consisting of chloroform/methanol/water (65/25/4, v/v) and quantified by comparison to standard curves of authentic standards. Following development in 100 ml of chloroform/acetone (9:1, v/v) for 30 min, cholesterol and fatty acids in crude lipid extracts (50–100 nmol of total phospholipid phosphate) were identified from TLC plates by comparison to known standards run in parallel on the same plates.

Statistics—The data from three separate experiments were pooled, analyzed by the Student test t, and expressed as mean ± S.E. A value of p < 0.05 was considered statistically significant.

RESULTS

Mouse and other small animal aortic endothelial cells are resistant to isolation by enzymatic treatment. Therefore, an explanting procedure was employed to generate primary cultures of endothelial cells. This procedure provides an efficient and reliable technique for the isolation and cultivation of murine aortic endothelial cells. The experimental conditions were modified and optimized from those originally reported. The outgrowth of aortic endothelial cells was enhanced by the removal of antibiotics from the plating medium. The contamination of other mixed cell types, including fibroblasts, was minimized by removal of the aortic rings prior to the time at which non-endothelial cells started to migrate from the aortic rings. The purity of the endothelial cell population was controlled by the addition of heparin, which promoted markedly endothelial cells growth by stimulating the activation of endothelial cell growth factor (16) and induced an anti-proliferative effect on smooth muscle cells by blocking the activity of platelet-derived growth factor (17).

The purity of the glycosphingolipid-enriched plasma membrane fractions, from MAECs obtained from wild-type and α-Gal A-null MAECs, was evaluated (Fig. 1). Caveolin 1, caveolin 2, and annexin II were highly enriched in the membrane fractions from both wild-type and null endothelial cells. Multimeric caveolins 1 and 2 were highly enriched in both wild-type and null cells. The levels of multimeric caveolin 1 were lower in the knock-out cells and decreased with age. The opposite trend was observed for caveolin 2. Annexin II levels were consistently higher in the raft fractions recovered from the knock-out cells.

The effect of serum on the induction of Gb3 in wild-type cultured cells was eliminated by gradually reducing the concentration of serum in the culture medium. Previously we demonstrated that total cellular Gb3 was absent in wild-type endothelial cells (7). After 4 days of culture in 10% FBS-RPMI medium, no Gb3 was detected in caveolar lipids extracted from wild-type MAECs as well (Fig. 2A, lane 1). However, under these experimental conditions, the presence of Gb3 in cultured α-Gal A-knock-out MAECs persisted and progressively increased in caveolar membrane lipids (Fig. 2A, lanes 2–4) as well as in total cellular lipids (Fig. 2B) as a function of the age of the mice. The Gb3 accumulation in α-Gal A-knock-out MAECs was retained in the cultured cells up to 5 passages without decrement (data not shown). The Gb3 content did decrease 10–20% at later passages (P6–P8). By mass analysis, it was observed that the accumulation of Gb3 in caveolar membranes isolated from cultured α-Gal A-knock-out MAECs was age-dependent (Fig. 2B) but gender-independent (data not shown).

At earlier ages (≤2 months) the Gb3 levels in caveolar membranes were comparable to levels measured as total cellular lipids. With increasing age (≥5 months), however, higher Gb3 was found in caveolar membranes when normalized as recovered phospholipid phosphate. The accumulation of Gb3 in caveolar membranes reached a plateau in cells from 5- to 6-month-old mice, whereas the plateau for total cellular Gb3 was observed in cells obtained from 4-month-old mice.

The surprisingly large increase of Gb3 in caveolar membranes raised a possibility that the content of other caveolar lipids, normally enriched within caveolar membranes, would change as well. GlcCer and LacCer are two biosynthetic precursors of Gb3. The levels of GlcCer in total cellular and caveolar membrane lipids of wild-type and α-Gal A-knock-out MAECs were compared by HPTLC charred with 8% cupric sulfate in 8%
phosphoric acid (Fig. 3). There was no statistical difference in the levels of GlcCer between in total cellular (Fig. 3A) and in caveolar membrane lipids (Fig. 3B) isolated from cultured wild-type MAECs. However, the levels of GlcCer in caveolar membrane lipids extracted from cultured α/Gal A-knock-out MAECs significantly changed in an age-dependent (Fig. 3B) and gender-independent manner (data not shown). The levels of GlcCer in both total cellular and caveolar membrane lipids of MAECs from younger Fabry mice (2 months of age) were markedly lower compared with wild-type cells. With increasing age the levels of GlcCer in the caveolar membranes of Fabry MAECs increased linearly. By 8 months of age, the GlcCer level in caveolar lipids of α/Gal A-knock-out MAECs was 25% higher than in those of wild-type MAECs. A plateau point for GlcCer accumulation in caveolar lipids of α/Gal A-knock-out MAECs could not be documented even in cells obtained from 8-month-old mice.

FIGURE 2. A, accumulation of Gb3 in caveolar membranes of cultured α/Gal A-knock-out MAECs. Caveolar fractions were isolated from cultured MAECs using a detergent-free method as described under "Experimental Procedures." Caveolar lipids were then extracted from caveolar fractions with chloroform/methanol/0.9% NaCl at the ratio of 1/2/0.8 (v/v). The neutral lipids were purified by base and acid hydrolysis and analyzed by HPTLC using two solvent systems of chloroform/methanol/water/acetate acid/NaOH (64/31/3/2/0.5, v/v). WT, wild-type MAECs; KO, α/Gal A-knock-out MAECs. B, comparison of age-dependent Gb3 accumulation in whole cellular lipids and caveolar lipids of cultured α/Gal A-knock-out MAECs. Lipids were extracted either directly from cultured MAECs or secondary from caveolar fractions isolated from cultured MAECs. The Gb3 levels in the lipid samples were quantified by lipid mass analysis as described above.

FIGURE 3. A, levels of GlcCer in whole cellular lipids of cultured wild-type and α/Gal A-knock-out MAECs. B, levels of GlcCer in caveolar lipids isolated from cultured wild-type and α/Gal A-knock-out MAECs. Equal amounts (100 nmol of total phospholipid) of either whole cellular or caveolar lipids were subjected to base-hydrolysis. The GlcCer in neutral lipids were separated by HPTLC run in a solvent mixture containing chloroform/methanol/water (65/25/4, v/v) and determined by compared with authentic GlcCer standards. WT, wild-type MAECs; KO, α/Gal A-knock-out MAECs.

FIGURE 4. Measurement of LacCer levels in whole cellular and caveolar lipids of cultured MAECs. Both whole cellular and caveolar lipids were extracted and normalized for 100 nmol of total phospholipid. After base-hydrolysis, lipids were analyzed by HPTLC as described in Fig. 2. A, LacCer levels in whole cellular lipids. B, LacCer levels in caveolar lipids. WT, wild-type MAECs; KO, α/Gal A-knock-out MAECs.
The levels of LacCer in total cellular (Fig. 4A) and caveolar membranes (Fig. 4B) were measured using a solvent system that consisted of chloroform/methanol/water (65/25/4, v/v), and determined according to a standard curve generated from known amounts of LacCer. Compared with stably expressed LacCer in caveolar membranes of wild-type MAECs, the levels of LacCer in caveolar membranes of /H9251-Gal A-knock-out MAECs increased with age and paralleled Gb3 accumulation. The linear increase of caveolar LacCer in /H9251-Gal A-knock-out MAECs also did not reach a plateau at 8-month-old Fabry mice.

Differences in globotetraosylceramide (Gb4), a downstream biosynthetic product of Gb3, were also observed when measured in the caveolar lipids in comparison to whole cell Gb4 (Fig. 5). The pattern of Gb4 change was different than that observed with GlcCer and LacCer. Gb4 increased in caveolar lipids of /-Gal A-knock-out MAECs and reached maximal levels in cells from 6-month-old mice. However, the amount of Gb4 in the caveolar lipids from MAECs obtained from Fabry mice older than 6 months decreased to wild-type levels, implicating the induction of an alternative pathway for metabolism.

It is well established that caveolae lipids not only are enriched in glycosphingolipids, but also in cholesterol. Thus, the levels of cholesterol in caveolar membrane and total cellular lipids of Fabry MAECs were compared with the levels of cholesterol in wild-type MAECs. With progressive deposition of Gb3 in total cellular and caveolar lipids, cholesterol levels in both lipid samples decreased gradually (Fig. 6A) as detected with a solvent system containing 90% chloroform and 10% acetic acid (v/v). At 8 months of age, the amount of cholesterol in the caveolae of /-Gal A-knock-out MAECs was 63% lower compared with that in caveolae of 2-month-old cells (Fig. 6B). The enrichment of fatty acids in caveolar lipids of cultured MAECs was an unexpected finding (Fig. 6A, lanes 6 and 7). However, most of the fatty acid increase was no longer observed in the caveolar lipids of cultured MAECs isolated from Fabry mice /H11350 6 months of age (Fig. 6A, lanes 8 and 9).

We previously reported that two pharmacological agents, recombinant human /-Gal A protein and the GlcCer synthase inhibitor d-t-EtDO-P4, depleted Gb3 from total cellular lipids of cultured /-Gal A-knock-out MAECs with different efficiencies (7). In this study, the effects of these two pharmacological strategies on the depletion of Gb3 present in caveolae of /-Gal A-knock-out MAECs were compared (Fig. 7A). The clearance of excessive Gb3 from caveolae by d-t-EtDO-P4 was readily observed. A single treatment with 0.3 /M d-t-EtDO-P4 for 24 h was as effective as 1 or 1.5 /M d-t-EtDO-P4 for 2 days in elim-
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Removing Gb3 by D-\textsuperscript{-}Gal A and D-\textsuperscript{-}EtDO-P\textsubscript{4} with a single dose of recombinant human \textit{α}-Gal A and D-\textsuperscript{-}EtDO-P\textsubscript{4} but decreased within 2 days in those cells treated with \textit{α}-Gal A.

**DISCUSSION**

Over 40 lysosomal storage diseases have been identified. Fourteen of these disorders involve the accumulation of sphingolipids. Duve (18) recognized that these diseases often resulted from the failure of lysosomal enzymes to function properly. Subsequently, it has been recognized that the molecular basis for lysosomal storage could result from the defective transport or lysosomal enzymes, defective lysosomal membrane transporters, and the lack of activator proteins that regulate lysosomal hydrolase activity (19). While much is known about the proximate molecular and genetic basis of these disorders, how individual diseases result in cellular and organ pathophysiology is significantly less well understood. Indeed, it has been difficult to understand how the common finding of intralysosomal metabolite accumulation results in such a wide range of phenotypes among the 40 different disorders.

Several hypotheses have been considered to explain the cellular pathology of lysosomal storage disease. These hypotheses include derangements in lysosomal stability, aberrant intracellular trafficking, altered gene expression with the activation of secondary biochemical pathways, and defects in intracellular signaling. An alternative hypothesis, namely that sphingolipids accumulate outside of the lysosomes, has received considerably less attention. Under this hypothesis, the cellular pathology results from the presence of abnormal levels of sphingolipids in membrane compartments external to the lysosome affecting the regulation of signaling processes, which are dependent on the local sphingolipid composition and levels.

Sphingolipids in addition to cholesterol are important components of caveolin-associated lipid rafts. Not only are these subsets of lipid rafts highly enriched in sphingomyelin and glycosphingolipids, but the endosomal trafficking of these rafts appears to be specifically regulated. For example, fluorescently tagged sphingolipids are recycled to the cell surface through early endosomes rather than cycled to late endosomes of lysosomes (20). Late endosomes or vacuoles in \textit{Saccharomyces cerevisiae} contain low amounts of sphingolipids and ergosterol (21). These and other findings suggest that sphingolipids are
Independent of these abnormalities, the effects of aberrant changes in sphingolipid content within caveolae should also be considered. Glycosphingolipids have long been recognized as important mediators of cell signaling responses at the plasma membrane. The activity of receptor tyrosine kinases, including the insulin receptor and epidermal growth factor receptor, is directly modulated by the associated ganglioside content. The activities of phospholipase C γ-1 and a variety of Src kinases can be similarly demonstrated to be dramatically affected by genetic or pharmacologic manipulations of cellular glycosphingolipids (9, 10). Recently, it has been reported that these changes may be mediated by the direct binding of tetraspanins to membrane glycosphingolipids as part of a larger structure termed a glycosynapse (23). These observations beg an important question, namely, do specific glycosphingolipids and changes in their relative concentrations regulate specific signaling phenomena?

In the present study we have studied the plasma membrane caveolin-associated lipid raft content in endothelial cells of wild-type and α-Gal A null mice. These cells may represent a useful model of Fabry disease, because they retain high levels of Gb3 even following serial passages. Importantly, we observed that the caveolar content of Gb3 is increased in the knock-out cells compared with wild-type cells. The change in Gb3 content is increased as a function of the age of the mice. Secondary changes are also observed in the levels of the Gb3 precursors GlcCer and LacCer. Caveolin-associated levels of these neutral glycosphingolipids are below those of the wild-type cells and increase with age. By 6–8 months of age the levels of these glycolipids are equal to or in excess of those in the wild-type cells. At this point in time, the levels of cholesterol decrease proportionately. By contrast, no change in sphingomyelin levels is observed.

The caveolar content of glycosphingolipids also changed following pharmacological treatment. Treatment of Fabry endothelial cells with α-Gal A and D-t EtDO-P4 were comparably effective in lowering the Gb3 content of the raft fractions. These data suggest that the turnover of Gb3 is sufficiently rapid to respond to acute changes in synthesis or degradation. The changes in glycosphingolipid content differed, however, between the two agents. The effect of D-t EtDO-P4 persisted for a considerably longer period of time compared with α-Gal A upon withdrawal of drug. The response to the two agents differed in additional potentially important respects. α-Gal A treatment resulted only in the reduction of Gb3. By contrast, GlcCer, LacCer, and G4 levels decreased when the GlcCer synthase inhibitor was used. Finally, although both agents partially reversed the loss of cholesterol in the caveolae, EtDO-P4 was more effective than α-Gal A in normalizing the cholesterol content and the effect was more persistent.

Collectively, these data suggest that both the composition and content of raft-associated lipids change dynamically in the endothelium of the α-Gal A null mouse. To the best of our knowledge, this is the first report of a change in plasma membrane caveolin-associated glycosphingolipids in a lysosomal storage disorder. However, the non-glycosphingolipid lipid composition in detergent-resistant membrane fractions has been studied in another mouse model of lysosomal storage dis-
ease, Niemann-Pick C. In a recent study the cholesterol content of plasma membranes was observed to be increased from hepatocytes of NPC−/− mice. The increased cholesterol impaired insulin signaling and was restored following cholesterol depletion (24). In other studies cholesterol levels have been reported to be increased, decreased, or unchanged (25–28).

In summary, this study demonstrates the age-dependent change of Gb3 and related globo series glycosphingolipids in the caveolin-associated lipid raft fractions of plasma membranes from the α-galactosidase A null mouse endothelial cells. The changes are rapid, complex, and reversible by agents that either block Gb3 synthesis or promote its degradation. The complexity of these changes will be an important variable in fully understanding the basis of the vascular pathology of Fabry disease and the pathophysiology of lysosomal storage diseases more generally.

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