Decreased Nitric Oxide Bioavailability in a Mouse Model of Fabry Disease

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ABSTRACT
Fabry disease is a lysosomal storage disorder that results in an accumulation of globotriaosylceramide in vascular tissue secondary to a deficiency in a-galactosidase A. The glycolipid-associated vasculopathy results in strokes and cardiac disease, but the basis for these complications is poorly understood. Recent studies in the a-galactosidase A–knockout mouse suggested that a decrease in nitric oxide (NO) bioavailability may play a role in the abnormal thrombosis, atherogenesis, and vasorelaxation that are characteristic of these mice. To understand better the association between impaired NO bioavailability and glycolipid accumulation, we studied a-galactosidase A–knockout mice or primary cultures of their aortic endothelial cells. Treatment of knockout mice with a potent inhibitor of glucosylceramide synthase reversed accumulation of globotriaosylceramide but failed to normalize the defect in vasorelaxation. Basal and insulin-stimulated endothelial NO synthase (eNOS) activities in endothelial cells derived from knockout mice were lower than those observed from wild-type mice; normalization of glycolipid only partially reversed this reduction in eNOS activity. The loss of eNOS activity associated with a decrease in high molecular weight caveolin oligomers in endothelial cells and isolated caveolae, suggesting a role for glycolipids in caveolin assembly. Finally, concentrations of ortho-tyrosine and nitrotyrosine in knockout endothelial cells were markedly elevated compared with wild-type endothelial cells. These findings are consistent with a loss of NO bioavailability, associated with eNOS uncoupling, in the a-galactosidase A–knockout mouse.


Anderson-Fabry disease is a rare lysosomal storage disorder that arises secondary to an X-linked inherited deficiency in the enzyme a-galactosidase A (Gla).1 As a consequence of this enzyme deficiency, levels of globotriaosylceramide (Gb3) accumulate in the vascular tissues and kidneys of affected patients. A major manifestation of this disease is a large and small vessel vasculopathy with premature death resulting from strokes and cardiac disease. In a recently reported analysis of the Fabry disease registry, 21% of men with Gla deficiency reported significant cardiovascular events.2 Of note is the finding that approximately 40% of male patients with Fabry disease experience cerebrovascular complications.3 The existence of a primary defect in endothelial function in patients with Fabry disease manifest by abnormalities in regional blood flow is supported by several clinical studies. Patients with Fabry disease are also reported to have abnormalities in regional cerebral blood flow at rest.4 These abnormalities are usually associated with hyperperfusion. In addition, a decrement in coronary artery reserve

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flow and impaired postischemic forearm perfusion have been reported.5

The Gla knockout mouse has provided a useful model for studying vascular disease. Although these mice do not display a spontaneous vascular phenotype, recent reports have detailed three inducible models of vasculopathy. These models include oxidant-stimulated thrombosis in response to activation of rose Bengal,6 accelerated atherogenesis in mice bred on apolipoprotein E null background,7 and impaired vascular reactivity in aortic rings in response to α adrenergic agonists and vasorelaxation in response to acetylcholine.8,9

Recently, our group has been studying the basis for the vasoreactive defect in the Gla knockout mouse.9 The defect in vascular responsiveness was observed to reside in the endothelium on the basis of three experimental observations. First, both the vasoconstrictive and vasorelaxation abnormalities were abolished when the endothelium was removed. Second, no difference between wild-type and Gla knockout mice was observed when relaxation was induced with sodium nitroprusside. Third, when aortic rings were exposed to low concentrations of ionomycin, no difference in calcium-induced vasorelaxation was observed between wild-type and null mice. These data suggest that the vasoreactive defects in the Gla knockout mice reside not only in the endothelium but also at the level of plasma membrane.

Although the thrombotic, atherogenic, and vasoreactive abnormalities seen in the Gla knockout mice may result from independent and disparate mechanisms, a common mechanism could account for the observed phenotypes, viz. the loss of endothelial nitric oxide synthase (eNOS) activity. The loss of normal NOS signaling has been associated with each of these phenotypes, and several mechanisms for the loss or impairment of normal eNOS activity have been identified. In this report, we studied the vasoreactivity of aortas in knockout mice in which Gb3 levels were reduced by a potent inhibitor of glucosylceramide synthase. We observed no statistically significant correction of the vasorelaxation in response to acetylcholine after normalization of Gb3 levels. We subsequently studied the expression, activity, and functional coupling of eNOS in the aortas and aortic endothelium of wild-type and Gla knockout mice to ascertain whether these might account for the partial response and the induced vascular phenotypes.

**RESULTS**

Gb3 Reduction by Systemic Administration of Glucosylceramide Synthase Inhibitor Fails to Reverse the Defect in Relaxation to Acetylcholine

To explore the association between elevated Gb3 and the vasorelaxation defect of the Gla−/0 null mice, we used the potent glucosylceramide synthase inhibitor D-threo-ethylenedioxyphenyl-2-palmitoylaminio-3-pyrrolidino-propanol (EtDO-P4). This small molecule inhibitor of glucosylceramide-based glycosphingolipid synthesis was designed and demonstrated by our group to reduce Gb3 potently in Gla−/0 mouse liver, kidney, and heart.10,11 Seventeen to 22-wk-old Gla−/0 mice were treated with 10 mg/kg per d inhibitor in sulfobutyl ether β-cyclodextrin (Captisol) carrier or with the carrier alone by daily intraperitoneal injections for 28 d.12 Twenty-four hours after the last injection, the aortas were isolated for Gb3 detection by immunofluorescence and contractility studies. When Gb3 was measured by Shiga toxin B subunit binding, we observed a near-complete reduction in the aortic Gb3 content (Figure 1). This reduction was evident throughout the aortic wall and at the luminal edge, suggesting that the inhibitor blocked glucosylceramide-based glycolipid synthesis throughout all levels of the artery.

Previously, we had demonstrated that when aortic rings were precontracted with phenylephrine to 80% of their maximal contraction, Gla−/0 aortic rings failed to relax to the same degree as those from Gla+/0 mice. When the vehicle- and inhibitor-treated mouse aortic rings were similarly studied, no significant improvement in the relaxation response was observed (Figure 2).

**Age-Dependent Decrease of High Molecular Mass Oligomerization of Caveolin-1 in Gla−/0, not Gla+/0, Mouse Aortas**

To understand better the molecular basis of the endothelial dysfunction in Gla−/0 mice, we compared the age-dependent expression of caveolin-1 and eNOS in wild-type and...
Gla−/0 mouse aortas. Freshly isolated mouse aortas were lysed in 1% Triton X-100 lysis buffer, and the clarified aortic lysates were subjected to a gradient SDS-PAGE separation. Caveolin-1 was detected with a polyclonal antibody directly against the N-terminus of mouse caveolin-1 (MSGGKDYDSEGHLYTVP-C). Under nonreducing (0% β-mercaptoethanol) and without heating, two high molecular mass homo-oligomers of caveolin-1 (>250 kD) were readily detected in Gla+/0 and younger Gla−/0 mouse aortas (Figure 3A, lanes 1 through 4); however, the detection of high molecular mass oligomers of caveolin-1 was significantly decreased in the aged aortas from 4- to 16-mo-old Gla−/0 mice (Figure 3A, lanes 5 through 8). The expression of 20-kD monomeric caveolin-1 was also decreased as a function of age. The high molecular mass oligomers were not detected under reducing conditions (Figure 3B).

Reduced Immunoreactivity of eNOS in Aged Gla−/0 but not Gla+/0 Mouse Aortas

We next analyzed the age-dependent immunoreactivities of eNOS in Gla+/0 and Gla−/0 mouse aortas. The aortic proteins were solubilized in 1% Triton X-100 lysis buffer mixed with loading buffer containing 1% β-mercaptoethanol; after heating at 80°C for 5 min, the samples were separated in SDS-PAGE with a 6 to 12% gradient. The immunoblot analysis revealed that in aged Gla−/0 mice (8 and 12 mo old), the expression of eNOS was significantly decreased compared with age-matched wild-type mouse aortas (Figure 4).

Co-localization of eNOS and Caveolin-1 in Mouse Aortic Extracts

To ascertain whether there was a correlation between the decreased expression of eNOS and caveolin-1, we performed immunoprecipitation studies on the aortas of Gla+/0 and Gla−/0 mice. Caveolin-1 expression was com-
pared in the aortas from 12- and 16-mo-old wild-type and knockout mice (Figure 5). The decrease in caveolin-1 expression was observed after immunoprecipitation of eNOS (Figure 5A), and, conversely, the decrease in eNOS expression was observed after the immunoprecipitation of caveolin-1 (Figure 5B). These data suggest that eNOS and caveolin-1 co-localize in the wild-type and knockout aortas and that the decreased expression of eNOS may result, in part, from the lower content of caveolin-1.

High Levels of Gb3 Accumulate in the Cultured Mouse Aortic Endothelial Cells Isolated from Aged Gla−/0, not Gla+/−, Mice

Our previous studies on the Gla−/0 mouse aortas suggested that the primary vascular reactivity defect resides in the endothelium. To explore further the basis for this defect and to determine whether changes in caveolin and eNOS expression could be localized to the endothelium, we isolated and grew primary cultures of mouse aortic endothelial cells (MAECs) as described in the Concise Methods section. Each batch of Gla+/0 or Gla−/0 MAECs was isolated and pooled from a total of 12 wild-type or null mice at 8 mo of age. At the third passage, the cells were grown on gelatin-coated glass coverslips and maintained in 10% FBS-RPMI 1640 medium for 3 d. Gla−/0 but not Gla+/0 MAECs expressed high levels of Gb3 as detected by verotoxin B subunit binding (Figure 6). The treatment of the MAECs with 200 nM EtDO-P4 for 48 h resulted in an almost complete elimination of the verotoxin binding. This finding is consistent with previous reports in which the Gb3 mass was significantly reduced by inhibitor treatment.11,13

Decreased Expression and Activity of eNOS in Cultured Primary MAECs Isolated from Aged Gla−/0

The expression and activity of eNOS was measured in MAECs from Gla+/0 and Gla−/0 mice of 4 or 8 mo of age. Cultured primary MAECs at passage 3 were grown to 80% confluence in 100-mm culture dishes for immunoblot analysis and in 60-mm dishes for measurements of eNOS activity. The concentration of serum in medium was gradually decreased from 20 to 0%, and endothelial growth factors were omitted for 12 h before eNOS was measured. With heating (80°C for 5 min) and nonreducing conditions, eNOS in cultured MAECs was not detected (data not shown). Under reducing conditions (1% β-mercaptoethanol) and in the absence of heating, the eNOS expression in cultured MAECs was detected with a polyclonal antibody against the C-terminal amino acids 1178 through 1228 of eNOS.

Figure 5. eNOS/caveolin-1 immunocomplex formation in the aortas of Gl+/0 and Gla−/0 mice. (A, a) Caveolin-1 co-immunoprecipitation with eNOS was compared between the aortas from Gl+/0 (lanes 3 and 7) and Gla−/0 mice (lanes 4 and 8). Caveolin-1 immunoblotting without immunoprecipitation (lanes 1 and 2) served as a positive control. Recovered caveolin-1 (lanes 5 and 6) after immunoprecipitation served as a second positive control. (b) The heavy chain of eNOS antibody [Ab (HC)] was detected with a goat anti-mouse Ig antibody (lanes 3 and 4, 7 and 8). (c) Ab (HC) was detected with a goat anti-rabbit Ig antibody (lanes 5 and 6). (B, top) eNOS co-immunoprecipitation with caveolin-1 was compared between the aortas from Gl+/0 (lane 3) and Gla−/0 mice (lane 4). eNOS immunoblotting without immunoprecipitation (lanes 1 and 2) served as a positive control. (Middle) The Ab (HC) was detected with a goat anti-rabbit Ig antibody (lanes 3 and 4). Aortic β-actin in A (d) and B (bottom) served as an internal loading control. The blots are representative of three independent experiments that yielded equivalent results.

Figure 6. STX binding and Gb3 accumulation in cultured Gal−/0 MAECs. MAECs grown on glass coverslips precoated with gelatin were overlain with purified STX-1 and subsequently with Alexa Fluor 546-conjugated secondary antibody. (A) Cultured Gla+/0 MAECs isolated from C57Bl/6 control mice at age of 8 mo. (B) Cultured Gla−/0 MAECs isolated from 8-mo-old mice. (C) Cultured Gla−/0 MAECs isolated from 8-mo-old mice treated for 48 h with 0.2 µM EtDO-P4.
1194 of mouse eNOS. Stable and consistent expression of eNOS was detected in cultured MAECs obtained from 2- to 8-mo-old wild-type mice (data not shown); however, the eNOS levels were significantly lower in cultured Gla−/0 MAECs from 4- and 8-mo-old Gla null mice (Figure 7A, lanes 5 and 7). The treatment of cultured Gla−/0 MAECs with EtDO-P4 restored partially the eNOS levels in the Gla-deficient cells (Figure 7A, lanes 6 and 8).

We next examined the eNOS expression in response to insulin in Gla−/0 MAECs from 4-mo-old mice. No significant difference was observed in the eNOS levels between unstimulated Gla−/0 MAECs and MAECs exposed to 100 nM insulin for 5 or 10 min (Figure 7B, lanes 1 to 3); however, insulin stimulation increased eNOS at 15 min (Figure 7B, lane 4). EtDO-P4 pretreatment (0.25 μM for 2 d) significantly increased eNOS levels in cultured Gla−/0 MAECs (Figure 7B, lane 5) compared with untreated Gla−/0 MAECs (Figure 7B,

Figure 7. Decreased eNOS expression and activity in cultured MAECs prepared from Gla−/0 mice. (A) eNOS was compared by Western blots of gels from wild-type and Gla−/0 MAECs. β-Actin levels were used as a control. Lanes 1 through 4, no change in eNOS levels in Gla+/- MAECs in response to insulin stimulation; lanes 5 and 7, reduced expression of eNOS in cultured Gla−/0 MAECs prepared from 4- and 8-mo-old mice; lanes 6 and 8, partial restoration of the immunoreactivity of eNOS in EtDO-P4–treated Gla−/0 MAECs isolated from 4- and 8-mo-old Fabry mice. (B) Immunoreactivity of eNOS in Gla−/0 MAECs from 4-mo-old Fabry mice stimulated with insulin for 0, 5, 10, and 15 min in the absence (lanes 1 through 4) and presence (lanes 5 through 8) of 0.25 μM EtDO-P4. Cellular β-actin served as a loading control. The blots are representative of three independent experiments with similar results. (C) eNOS activity in cultured wild-type and Gla−/0 MAECs isolated from 4- and 8-mo-old mice. Data are means ± SE (n = 6).

Figure 8. Decreased formation of high molecular mass oligomers of caveolin-1 in cultured Gla−/0 MAECs. (A) Representative immunoblots of high and low molecular weight caveolin-1 in wild-type and Gla−/0 MAECs cultured from 4- and 8-mo-old mice. Gla−/0 cells were also treated for 48 h with 0.25 μM EtDO-P4. (B) Densitometric data from the immunoblots (n = 3). Data are means ± SE.
loss of monomeric and oligomeric caveolin-1 reflecting the findings observed in intact aortas. High molecular weight oligomers of caveolin-1 were markedly decreased in the Gla−/0 MAECs compared with those from wild-type mice (Figure 8A). Repeated measurements of caveolin-1 expression demonstrated an age-dependent decrease in both monomeric and oligomeric forms of caveolin-1. Gb3 depletion with 250 nM EtDO-P4 only partially increased caveolin recovery in the MAECs from 8-mo-old knockout mice without any significant effect in the cells from the 4-mo-old mice (Figure 8B).

**Caveolar Localization of Caveolin-1 and eNOS in Cultured MAECs**

Previously, we examined the compartmentalization of glycosphingolipids and caveolin-1 in caveolar fractions of cultured Gla−/0 and Gla+/0 MAECs. Caveolin-1 was observed to decrease in cultured Gla−/0 MAECs as a function of glycosphingolipid content and age. The recovery of eNOS in caveolae was also evaluated. No difference in eNOS levels was observed in caveolar fractions in MAECs isolated from 2-mo-old Gla−/0 and Gla+/0 mice; however, an age-dependent decrease in caveolar eNOS was observed in cells from 4- and 8-mo-old knockout mice compared with their wild-type counterparts (Figure 9A). Monomeric and oligomeric caveolin-1 was assayed from 8-mo-old mouse MAECs. A decrement in both high and low molecular weight caveolin-1 was observed in the Gla−/0 MAECs compared with those from wild-type mice (Figure 8A). Repeated measurements of caveolin-1 expression demonstrated an age-dependent decrease in both monomeric and oligomeric forms of caveolin-1. Gb3 depletion with 250 nM EtDO-P4 only partially increased caveolin recovery in the MAECs from 8-mo-old knockout mice without any significant effect in the cells from the 4-mo-old mice (Figure 8B).

**eNOS Uncoupling in Gla−/0 MAECs**

We sought to determine whether the decrease in eNOS activity was accompanied by an uncoupling of eNOS. Uncoupling of the enzyme results in the formation of the highly reactive oxidant peroxynitrite, which forms when NO reacts with superoxide. Previous studies demonstrated that ortho-tyrosine and nitrotyrosine are markers of peroxynitrite-mediated oxidation. To determine whether proteins from endothelial cells contain oxidized amino acid products of peroxynitrite oxidation, we isolated endothelial cells from Gla+/0 and Gla−/0 aortas. After delipidating the cells and hydrolysis with acid, we isolated amino acids. The content of the two oxidized amino acids—ortho-tyrosine and 3-nitrotyrosine—we analyzed “molecular signatures” characteristic of peroxynitrite-mediated

**Decreased Expression of Caveolin-1 in Aged Gla−/0 MAECs**

We performed immunoblots on MAECs from 4- and 8-mo-old Gla−/0 and Gla+/0 mice to determine whether there was a
oxidation by isotope dilution liquid chromatography tandem mass spectrometry (Figure 10). The samples from the endothelial cells of Gla−/0 mice contained 90% more ortho-tyrosine than those from the wild-type animals (Gla−/0, 63.8 ± 8.6 μmol/mol phenylalanine [the precursor of ortho-tyrosine]; Gla+/0, 33.7 ± 3.0 μmol/mol phenylalanine; n = 3 per group). Similarly, levels of nitrotyrosine were 72% greater in the Gla−/0 endothelial cells (Gla−/0, 93.70 ± 18.60 μmol/mol tyrosine [the precursor of nitrotyrosine]; Gla+/0, 54.37 ± 1.40 μmol/mol tyrosine; n = 3 per group). The marked elevation of both of these oxidized amino acids is consistent with peroxynitrite oxidation of proteins in the endothelial cells. The increases in ortho-tyrosine and nitrotyrosine were not corrected when the Gb3 levels in the cells were normalized with treatment with EtDO-P4. These observations suggest that the eNOS is uncoupled in this in vitro model.

**DISCUSSION**

Previous in vivo studies with the Gla null mouse demonstrated three primary phenotypes when compared with strain-specific wild-type mice. These include enhanced thrombosis secondary to oxidant-induced endothelial injury, accelerated atherogenesis when mice are bred on an apolipoprotein E null background, and impaired vasorelaxation to acetylcholine.8

Contrasted aortic rings. Although these phenotypes may result from disparate and complex abnormalities, a potential common mechanism linking these abnormalities is the decreased bioavailability of NO. There are four principal causes of diminished NO bioactivity.14 These causes include decreased expression or activity of eNOS, uncoupling of eNOS, enhanced breakdown or scavenging of NO, and impaired transmission of NO-mediated signaling processes. In these studies, we considered the possibility that decreased NO bioavailability might partially explain these vascular phenotypes by first measuring caveolin-1 and eNOS expression in the aortas of the Gla null mice and their isogenic littermates.

To probe this possibility, Gla null mice were treated with a potent inhibitor of glucosylceramide synthase. EtDO-P4 potently reversed the Gb3 accumulation in aortic rings (and cultured endothelial cells) but did not reverse the vascular reactivity abnormality observed in the Gla knockout mouse aortic rings. The failure to correct the vasorelaxation defect is contrary to a previous report using N-butyldeoxynojirimycin. In that study, the inhibitor had minimal effects in lowering aortic Gb3 content but partially improved the vasodilatory response to acetylcholine.8

Our recently reported studies comparing aortic ring reactivity Gla null with wild-type mice demonstrated that the vascular defect resided within the endothelium of the aortas; therefore, to probe further a possible difference in NO bioavailability, we studied primary cultures of aortic endothelial cells from 4- and 8-mo-old mice. A decrease in basal and insulin-stimulated eNOS activity was observed in the Gla null cells compared with the wild-type MAECs. This decrement in NO formation was paralleled by similar decreases in caveolin-1 and eNOS expression. When Gb3 levels were lowered by exposure of the MAECs to EtDO-P4, a potent inhibitor of glucosylceramide synthase, we observed a partial but incomplete reversal of the defect in insulin stimulated NO formation, consistent with the in vivo observations; however, when the caveolar fractions were isolated from these cells and probed for both caveolin-1 and eNOS, we observed no change in the recovery of either protein.

eNOS synthesizes NO in endothelial cells, and its uncoupling has been described in various conditions, including diabetes, hypertension, and hypercholesterolemia. Under those conditions, eNOS transfers electrons to molecular oxygen, generating superoxide. Several mechanisms have been proposed for this uncoupling. One important mechanism involves oxidation of its co-factor, BH4.15 An alternative mechanism for uncoupling eNOS involves overproduction of angiotensin II, which can induce dihydrofolate reductase deficiency. Because dihydrofolate reductase maintains BH4 in its reduced form, its deficiency uncouples eNOS.16

When both NO and superoxide are generated by eNOS, it can result in the formation of the highly reactive oxidant peroxynitrite.17 Our data demonstrating marked elevations of protein-bound ortho-tyrosine and nitrotyrosine in MAECs isolated from Gla null mice is consistent with peroxynitritle-
induced protein damage. In addition to eNOS uncoupling, other cellular sources of excess superoxide production include mitochondria and NADPH oxidase. Peroxynitrite can be generated in the presence of NO under these conditions as well. The effects of Gla deficiency on these pathways remain to be elucidated. Peroxynitrite once generated can also oxidize BH₄ and perpetuate eNOS uncoupling; however, the mechanism by which elevated Gb3 results in aberrant eNOS function is not known. Posttranscriptional events are important for eNOS function. These events include the phosphorylation of the enzyme by one of several kinases, the routing of the enzyme to the caveolus, proper dissociation of eNOS from caveolin-1, and the availability of co-factors and substrate. Among the phosphorylation events are the phosphorylation of serine, threonine, and tyrosine residues, primarily located in the reductase domain of eNOS. The localization of eNOS to caveolae is also an important regulatory mechanism. The association of eNOS with these specialized lipid rafts occurs through palmitoylation and myristoylation sites and seems to depend on the lipid composition of the caveolae. Cholesterol and sphingolipids are critical components of lipid rafts and at least the former lipid is believed to be critical for caveolus formation. Approximately 100 to 200 caveolin molecules are present in each caveolus, most in an oligomeric form. In general, the anchorage of eNOS to caveolins results in the inactivation of the enzyme, and the dissociation results in activation. The consequences of eNOS-caveolin dissociation for signal transduction have been previously reviewed.

The importance of caveolar lipid is based primarily 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; however, the role of other caveolar lipid components in eNOS redistribution, most notable sphingomyelin and glycosphingolipids, does not seem to have been studied.

In this study, we observed a decrease in high molecular weight caveolin-1 oligomers observed in the Gla null mouse endothelium. This finding suggests that Gb3 may regulate the assembly or maintenance of caveolar structure. This regulation may be direct, conceivably as a result of a direct interaction between the glycosphingolipid and caveolin-1, or indirect secondary to the displacement of caveolar lipids that are normally present. The displacement of cholesterol by Gb3 in Gla null endothelial cells has been reported.

Caveolins are synthesized on the endoplasmic reticulum and transit through the Golgi complex before being trafficked to the plasma membrane. At some point during this synthetic route, caveolins change from a monomeric form to an oligomeric form. Studies using a caveolin-1–GFP fusion construct are consistent with the formation of the caveolae in the Golgi complex. Cholesterol seems to be important for this process. The addition of cholesterol seems to decrease the transit time through the Golgi complex. Cholesterol binds directly to caveolin-1 as demonstrated by photoactivatable cross-linking studies, plasma membrane cholesterol enrichment in cells expressing caveolin-1, and numerous structural studies defining cholesterol-binding motifs. Whether glycosphingolipids such as Gb3 also bind caveolin-1 directly and are important for caveolin assembly, trafficking, and structure is unknown but clearly worthy of further investigation.

In summary, direct measurements of eNOS activity and caveolin-1 oligomerization in aortic endothelial cells from Gla null mice demonstrate a loss of activity consistent with decreased NO bioavailability. The loss of NO, through either decreased production by eNOS or scavenging through the formation of superoxide and conversion to peroxynitrite, may account for aberrant vascular phenotypes in the Gla null mouse model that include atherogenesis, thrombosis, and impaired relaxation.

**CONCISE METHODS**

**Reagents**

Polyclonal anti-eNOS and anti–caveolin-1 antibodies were obtained from Abcam (Cambridge, MA), and a mAb directly against eNOS was from BD Transduction Laboratories (Lexington, KY). Shiga toxin-1 (STX) and anti-STX mAb (13C4) were obtained from Toxin Technology (Sarasota, FL). Monoclonal anti–β-actin antibody P2714 (a protease inhibitor cocktail with a broad specificity for the inhibition of serine, cysteine, and metalloproteases) and a 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), a water-soluble and more stable form of PMSF, were purchased from Sigma-Aldrich (St. Louis, MO). The glucosylceramide synthase inhibitor EtDO-P4 was designed and synthesized in our laboratory as described previously.

**Animals and Genotyping**

Gla-deficient mice, originally developed by Ohshima et al., and wild-type C57BL/6 mice were bred, housed, and maintained in the animal facility of the University of Michigan as described previously. The mouse genotypes were routinely confirmed using a simple, one-step PCR procedure. Genomic DNA was prepared from mouse tails by using DirectPCR Lysis Reagent (Viagen Biotech, Los Angeles, CA) following the instructions provided by the supplier. Crude DNA released in DirectPCR reagent was directly subjected to PCR amplification without further purification because this reagent inhibits the certain compounds that interfere with PCR reactions in animal tissues. The sequences of designed primers and PCR conditions for the Gla gene were as follows: forward primer 5’-ACTGGGTATCCTGGCTCTATCC-3’, reverse primer 5’-GATCTACGCCAGCACAGAAATG-3’, and primer for PGKneo sequence 5’-TCCATCTGCACGAGACTAGT-3’. The PCR reaction mixture (25 μl) contained 0.2 μM of each primer, 1X PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂, 0.2 mM dNTPs, 1.0 U Taq DNA polymerase, and 0.5 μl of genomic DNA (0.2 to 0.3 μg). Thirty cycles were performed for the PCR amplification. The cycling temperatures and
timings for Gla gene were 94°C for 30 s, 60°C for 30 s, and 68°C for 60 s. The generated PCR products—a 550-bp fragment for wild-type and an 885-bp fragment for knockout Gla, respectively—were separated on a 1.5% agarose gel. Animal studies were performed under the review of the University of Michigan committee on the use and care of animals and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MAEC Cultures
Primary MAECs were isolated, cultured, and maintained as described previously.13 Cultured MAECs were either studied at passages 3 to 4 or frozen at density of 1.0 to 1.5 × 10⁶ cells/ml in frozen medium consisting of 50% FBS, 10% DMSO, and 40% RPMI-1640. After thawing at 37°C for 2 to 3 min, frozen MAECs were recovered in RPMI-1640 medium supplemented with 15% FBS only for 3 to 4 h to allow cells to adhere to the culture dishes or preincubated in RPMI-1640 medium containing 15% FBS, 2 mM L-glutamine and 1× non-essential amino acid for overnight, if necessary. Recovery medium and unattached cells were then removed by replacement with RPMI-1640 medium containing 15% FBS, 2 mM L-glutamine, 1× nonessential amino acid, 0.05 mg/ml endothelial cell growth supplement (ECGS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.05 mg/ml heparin. Under those conditions, the recovery rate of frozen MAECs was usually >90%.

Aortic and MAEC Lysates
Aortas of Gla knockout and wild-type mice at ages 2 to 20 mo were isolated as described previously.13 Each aorta, gently dissected from fat and connective tissue in RPMI-1640 medium, was minced in 0.4 ml of ice-cold lysis buffer (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 2 mM EDTA, 2 mM Na₃VO₄, 20 mM NaF, 1% Triton X-100, 100 mM glycerol, 5% P2714, and 1 mM AEBSF) for 10 min at 4°C. Tissue debris was removed by centrifugation at 12,000 g for 10 min. The cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C. Tissue debris was removed by centrifugation at 12,000 × g for 10 min at 4°C. MAEC lysates were prepared from cultured wild-type and Fabry MAECs (passage 3) either treated or untreated with EtDO-P4 (0.25 µM) for 2 d. After removal of serum and growth factors, MAECs were stimulated with insulin (100 nM) for the indicated durations. The cells in 100-mm culture dishes were then lysed in 0.5 ml of 1% Triton X-100 lysis buffer as described already for 10 min at 4°C and collected by scraping. After a brief sonication (3 × 1 s), the cell debris was precipitated by centrifugation at 12,000 g for 10 min. The clarified aortic and MAEC lysates were subjected to immunoblot analysis after the determination of protein concentration by BCA assay using BSA as a standard.

Immunoprecipitation and Immunoblotting
For immunoprecipitation, equal amounts of aortic lysate proteins (100 to 150 µg) were first incubated with a polyclonal anti– caveolin-1 antibody (1 µg/ml) or with a monoclonal anti-eNOS antibody (2 µg/ml) overnight at 4°C with gentle rotation and subsequently incubated with protein A agarose beads (50 µl of a 50% suspension) for another 2 h at 4°C. The protein A agarose beads were then centrifuged at 7000 × g for 20 s and washed three times with a washing buffer consisting of 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM Na₂VO₄. The recovered immunocomplexes were dissolved in SDS sample buffer containing 0.5% β-mercaptoethanol and separated in SDS-PAGE with a 7 to 13% gradient. Immunoblots were performed as described previously.34 Equal amounts of either aortic or MAEC lysates were directly resolved by a 7 to 13% gradient SDS-PAGE. After membrane transfer, the nylon membranes were blocked in 5% skim milk in TBS buffer (20 mM Tris-HCl [pH 7.6] and 150 mM NaCl) for 1 h at room temperature and probed with selected antibodies. The immunoreactive bands were detected with the ECL-plus system (PerkinElmer Life Sciences, Waltham, MA) and quantified by densitometry using NIH Image 1.62 software (Bethesda, MD).

eNOS Activity Measurements
Enzyme activities of eNOS in cultured MAECs were determined as the formation of [³H]L-citrulline from [³H]L-arginine by adopting a protocol with high recovery rate of [³H]L-citrulline developed and described by Xiao et al.35 Briefly, MAECs (passage 3) untreated or treated with EtDO-P4 (2 d) were cultured in 60-mm dishes to 70 to 75% confluence. ECGS was first withdrawn from culture medium for 12 h. The concentration of FBS in the culture medium was then gradually reduced from 10 to 0%. Subsequently, cells were preincubated in 3 ml of L-arginine–deficient, serum-free, and ECGS-omitted RPMI-1640 for 2 h at 37°C. At a final 15 min period, cells were stimulated either with insulin (100 nM) or with buffer. The preincubation was stopped by removing the medium followed by washing once with 2 ml of Krebs-HEPES buffer consisting of 25.0 mM HEPES (pH 7.40, 37°C), 140.0 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM Na₂HPO₄, 25.0 mM NaHCO₃, and 5.5 mM glucose. Cells were then incubated in Krebs-HEPES buffer (2 ml), and the reaction was initiated by addition of 3.3 µCi of [³H]L-arginine (specific activity 59 Ci/mmol) diluted with 10 µM unlabeled L-arginine. After a 30-min incubation at 37°C, the reaction was terminated by removing the reaction buffer and rapidly rinsing the cells with 2 ml of ice-cold PBS containing 5 mM unlabeled L-arginine and 4 mM EDTA three times. Washed cells were solubilized but not denatured in 1.0 ml of 1% Triton X-100. Two aliquots (50 µl) were withdrawn for quantification of the total protein content and total cellular tritium incorporation. Two portions of cell lysate (0.3 ml) were taken, and each was mixed with 0.7 ml of 1:1 (vol/vol) H₂O/AG 50WX8-400 cation exchange resin (Na+ form [pH 5.5]) to remove unconverted [³H]L-arginine. After a 5-min mixing, the samples were centrifuged at 2000 × g for 2 min. The neutrally charged [³H]L-citrulline in the supernatants was collected, measured for radioactivity in a liquid scintillation counter, normalized to protein concentration, and expressed as pmol/min per mg protein. The samples were incubated in the presence of the competitive eNOS inhibitor, N⁶-nitro-L-arginine methyl ester (L-NAME), to account for nonspecific activity. The L-NAME–inhibitable (2 mM) activity was determined as specific eNOS activity.

Caveolae Isolation
Caveolar fractions were isolated from cultured MAECs either untreated or treated with EtDO-P4 at indicated times and concentrations by using a nondetergent method taking advantage of the unique buoyant density of caveolar membrane as described previously.36
Analysis of Oxidized Amino Acids

Cells were washed with ice-cold antioxidant buffer (100 μM diethylene tetramino pentaacetic acid [metal chelator], 50 μM butylated hydroxytoluene [lipid-soluble antioxidant], 1% [vol/vol] ethanol, and 10 mM 3-amino-1,2,4-triazole [peroxidase inhibitor]) in 50 mM sodium phosphate buffer [pH 7.4]). Cellular proteins were precipitated with ice-cold TCA (10% vol/vol), collected by centrifugation, washed with 10% TCA, and delipidated twice with water/methanol/water-washed diethyl ether (1:3:7; vol/vol/vol). Isotopically labeled internal standards were added, and samples were hydrolyzed with 4 N methanolic sulfonic acid. All samples were manually injected using an on column injector and a Hewlett Packard 6890 gas chromatograph equipped with a 15-m DB-5 capillary column (0.25 mm id, 0.33-μm film thickness; J&W Scientific, Folsom, CA) interfaced with a Hewlett Packard (Palo Alto, CA) 5973 mass detector. The t-butyl dimethylsilyl derivatives of amino acids were quantified by selected ion monitoring, using isotope dilution negative-ion chemical ionization gas chromatography mass spectrometry as described previously. Results are normalized to protein content of tyrosine, the precursor of 3-nitrotyrosine, or phenylalanine, the precursor for ortho-tyrosine.

Fluorescence Microscopy

Wild-type and Gla null MAECs at passage 3 were grown on glass coverslips precoated with 1% gelatin to 80% confluence. After washing twice with cold PBS, the cells were fixed with 1 ml of 4% cold fresh-prepared paraformaldehyde for 5 min at 4°C. The fixative was then removed by washing with ice-cold PBS, and cells were incubated with diluted STX (1:50) for 1 h at room temperature. The cells were subsequently blotted with anti-STX (13C4) mAb at 1:50 dilution for 1 h at room temperature and stained with goat anti-mouse antibody subsequently blotted with anti-STX (13C4) mAb at 1:50 dilution for 1 h at room temperature. The mounted coverslips were viewed under fluorescence microscopy and photographed as described previously.

Statistical Analysis

The data were collected from three or four individual experiments, analyzed by the t test and expressed as mean ± SEM. The differences between control and treated samples were considered statistically significant at P < 0.05.

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DISCLOSURES

J.A.S. is an inventor of D-threo-ethylendioxyphenyl-P4. The University of Michigan holds the patent to this compound and has licensed this inhibitor to Genzyme Corporation.

REFERENCES


