Endothelial nitric oxide synthase uncoupling and microvascular dysfunction in the mesentery of mice deficient in α-galactosidase A

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Kang JJ, Shu L, Park JL, Shayman JA, Bodary PF. Endothelial nitric oxide synthase uncoupling and microvascular dysfunction in the mesentery of mice deficient in α-galactosidase A, Am J Physiol Gastrointest Liver Physiol 306: G140–G146, 2014. First published November 14, 2013; doi:10.1152/ajpgi.00185.2013.—A defect in the gene for the lysosomal enzyme α-galactosidase A (Gla) results in globotriaosylceramide (Gb3) accumulation in Fabry disease and leads to premature death from cardiac and cerebrovascular events. However, gastrointestinal symptoms are often first observed during childhood in these patients and are not well understood. In this study, we demonstrate an age-dependent microvasculopathy of the mesenteric artery (MA) in a murine model of Fabry disease (Gla-knockout mice) resulting from dysregulation of the vascular homeostatic enzyme endothelial nitric oxide synthase (eNOS). The progressive accumulation of Gb3 in the MA was confirmed by thin-layer chromatographic analysis. A total absence of endothelium-dependent dilation was observed in MAs from mice at 8 mo of age, while suppression of ACh-mediated vasodilation was evident from 2 mo of age. Endothelium-independent dilation with sodium nitroprusside was normal compared with age-matched wild-type mice. The microvascular defect in MAs from Fabry mice was endothelium-dependent and associated with suppression of the active homodimer of eNOS. Phosphorylation of eNOS at the major activation site (Ser1179) was significantly downregulated, while phosphorylation at the major inhibitory site (Thr495) was remarkably enhanced in MAs from aged Fabry mice. These profound alterations in eNOS bioavailability at 8 mo of age were observed in parallel with high levels of 3-nitrotyrosine, suggesting increased reactive oxygen species along with eNOS uncoupling in this vascular bed. Overall, the mesenteric microvessels in the setting of Fabry disease were observed to have an early and profound endothelial dysfunction associated with elevated reactive oxygen species and decreased nitric oxide bioavailability.

Fabry disease; glycosphingolipids; renal failure; myograph; reactive nitrogen species

FABRY DISEASE is an X-linked glycosphingolipid disorder caused by deficiency or absence of activity of the lysosomal enzyme α-galactosidase A (Gla) (3, 12). The loss of Gla leads to the progressive accumulation of neutral glycosphingolipids with terminal Galα1-4Gal linkages, including globotriaosylceramide (Gb3), galabiosylceramide, and globotriaosylsphingosine. A primary site of glycosphingolipid accumulation is the vascular endothelium, although glycosphingolipid accumulation occurs in various other organ systems throughout the body (6, 23). Premature life-threatening complications arise from involvement of the brain, heart, and kidneys and include cerebrovascular accidents, myocardial infarction, and progressive renal insufficiency (7). Affected hemizygous males and female carriers suffer from Fabry disease-related complications (14, 25).

The vascular complications of Gla deficiency have been the primary focus of clinical and mechanistic studies of this rare disease. However, the earliest and most common symptoms of Fabry disease are gastrointestinal and include abdominal pain, constipation, diarrhea, and nausea (7, 20). Because these symptoms are common, the diagnosis of Fabry disease is often delayed (20).

Clinical and experimental studies have focused on the hypothesis that endothelial dysfunction may underlie the macrovascular and microvascular complications associated with Fabry disease. The Gla-knockout mouse can be used to characterize and study inducible models of vasculopathy. Robust vascular phenotypes, including accelerated atherogenesis and thrombus formation following photochemical injury, have been reported in these mice (2, 5). In isolated aortic rings, Gb3 accumulation in the endothelium is linked to a defect in endothelial nitric oxide (NO)-mediated vasodilation (17). These and other findings are consistent with a Gb3-dependent loss of NO bioavailability as a common mechanism linking atherothrombosis, thrombosis, and impaired vasorelaxation.

The purpose of this study was to determine whether the vascular phenotype in the Gla-knockout mouse extends beyond the aorta and carotid vasculature. Specifically, an age-dependent mesenteric artery (MA) phenotype was characterized in Gla-deficient mice. Age-dependent Gb3 accumulation, in conjunction with a profound loss of microvascular reactivity and increased endothelial NO synthase (eNOS) uncoupling, was observed in MAs of Gla-deficient mice.

METHODS

Mice. A breeding colony of Gla-null mice was established from mice provided by Ashok Kulkarni (National Institutes of Health). The knockout mice (129/SvJxC57BL/6) were originally generated by replacement of the Gla gene with a neomycin resistance (neo) sequence within a portion of the exon 3 and intron 4 region (16). These mice were backcrossed a minimum of six generations to the C57BL/6J strain. The Gla-null and wild-type (WT) C57BL/6 mice were maintained in the University of Michigan animal facility under standard conditions. All animal experiments were performed according to a protocol approved by the University of Michigan Committee on the Use and Care of Laboratory Animals.

Vascular reactivity experiments. Two- to 9-mo-old mice were euthanized with an injection of pentobarbital sodium (66.5 mg/kg ip). A segment of small intestine was removed and placed in a dissection petri dish filled with cold physiological salt solution (mmol/l: 130 NaCl, 4.7 KCl, 1.18 KH2PO4, 1.17 MgSO4, 1.6 CaCl2, 14.9 NaHCO3, 5.5 dextrose, and 0.03 CaNa2 EDTA). Second-order MAs (2–3 mm

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long) were carefully dissected, and connective tissue surrounding the arteries was removed. The individual vessel segment was mounted on glass cannulas in a pressure-myograph system (model 110P, Danish Myo Technology, Aarhus, Denmark). Vessel diameter was monitored and analyzed digitally in real time (DVM Myograph Acquisition Suite 6.2, Danish Myo Technology). In studies to determine ACh-mediated relaxation without endothelium, the endothelium was denuded during the mounting procedure by exposure of endothelial cells to an air bubble for 30 s. Mounted MAs were bathed with warmed (37°C) and aerated (95% O2-5% CO2) physiological salt solution. MAs were pressurized at 20 mmHg, and the pressure was increased 10 mmHg every 5 min until it reached 60 mmHg. The vessels were then equilibrated for 60 min. Prior to ACh- and sodium nitroprusside (SNP)-mediated vascular reactivity studies, the vessels were subjected to osmotically balanced high-potassium physiological salt solution (mmol/l: 14.7 NaCl, 100 KCl, 1.18 KH2PO4, 1.17 MgSO4, 2.7 CaCl2, 14.9 NaHCO3, 5.5 dextrose, and 0.03 CaNa2 EDTA) and 10^-2-10^-4 M norepinephrine (NE) with washes between each contraction. The vessel was preconstricted with NE (10^-5 M). Subsequently, ACh (10^-5-10^-3 M) or SNP (10^-8-10^-4 M) was added cumulatively to the bath for examination of endothelium-dependent (ACh) or endothelium-independent (SNP) relaxation. All chemicals used in the vascular reactivity study were purchased from Sigma Chemical (St. Louis, MO).

Reagents. Gb3 was purchased from Matreya (Pleasant Gap, PA); the phosphatase/protease inhibitors P2714, P0044, and P5726 from Sigma-Aldrich (St. Louis, MO); mouse anti-human eNOS and mouse anti-human 3-nitrotyrosine monoclonal antibodies from Abcam (Cambridge, MA); rabbit anti-human phosphorylated (Thr1179) eNOS polyclonal antibody from Cell Signaling Technology (Danvers, MA); rabbit anti-bovine phosphorylated (Ser1179) eNOS polyclonal antibody from Life Technologies (Grand Island, NY); and the ECL Plus system from PerkinElmer Life Sciences (Waltham, MA).

Tissue lipid extraction and Gb3 analysis. Frozen MA tissues, dissected from 1- to 12-mo-old WT and Gla-null mice, were thawed in 0.8 ml of ice-cold sucrose buffer (250 mM sucrose, pH 7.4, 10 mM HEPES, and 1 mM EDTA) per sample and homogenized with a TRI-R homogenizer for 20 s at 5% vessel. To determine whether the responsiveness of the smooth muscle layer to NO is different between MAs from WT and Gla-null mice, SNP, a NO donor, was added at increasing concentrations. SNP induced an endothelium-independent vasodilation in vessels from the 8-mo-old mice revealing a greater sensitivity at lower concentrations of SNP (80.5% vs. 5.0%) in WT than Gla-null mice (Fig. 3A). MAs from 8-mo-old WT mice relaxed in a concentration-dependent manner following exposure to ACh. Relaxation to the highest concentration of ACh in MAs from 8-mo-old WT mice (53.4 ± 9.2%) was comparable to that in MAs from WT 2-mo-old mice. By contrast, MAs from age-matched Gla-null mice were nearly devoid of ACh-mediated dilation (1.5 ± 5.8%, P < 0.001; Fig. 2B).

SNP-mediated relaxation in endothelium-intact MAs. The difference in SNP-mediated relaxation could be secondary to a functional defect in the endothelium or smooth muscle cells. To determine whether the responsiveness of the smooth muscle layer to NO is different between MAs from WT and Gla-null mice, SNP, a NO donor, was added at increasing concentrations. SNP induced a endothelium-independent vasodilation in MAs from WT and Gla-knockout mice. At 2 mo of age, the maximum relaxation to SNP in MAs from WT mice (78.2 ± 2.2%) was not statistically different from that in MAs from Gla-null mice (84.2 ± 5.4%; Fig. 3A). The maximum relaxation in MAs at 8 mo of age was also not different between WT (80.5 ± 5.0%) and Gla-null (69.6 ± 4.3%) mice. However, the SNP responsiveness in vessels from the 8-mo-old mice revealed a greater sensitivity at lower concentrations of SNP (10^-7-10^-6 M) in WT than Gla-null mice (Fig. 3B).

ACh- and SNP-mediated dilation in endothelium-denuded MAs. It was next determined whether the attenuation of ACh-mediated relaxation in the Gla-null mice was endothelium-dependent. The endothelium of the MAs was removed before determination of the ACh and SNP dose responses. As expected, denuding of the MA endothelium inhibited any measurable vasodilation in response to ACh in vessels from WT or
Gla-deficient mice (Fig. 4A). SNP induced a concentration-dependent vasodilation in both groups, and the maximum relaxation did not differ between WT (75.9 ± 3.2%) and Gla-null (74.4 ± 5.7%) mice (Fig. 4B).

Decreased eNOS homodimers and monomers in MAs from Gla-null mice. Next, the basis for the decrease in NO bioavailability was evaluated. Dimerization of eNOS is required for catalytic activity and NO production (27). Two forms of eNOS, monomers and homodimers, can be measured by PAGE under reducing or nonreducing conditions. A decrease in eNOS dimers and monomers was observed at 8 mo of age in MAs from Gla-null mice compared with WT mice (Fig. 5A). This difference was confirmed with repeated measurements (Fig. 5B). The 53% decrease in homodimer expression in MAs from Gla-knockout mice compared with MAs from WT mice (eNOS-to-actin ratio of 1.39 ± 0.08 vs. 0.66 ± 0.12) was statistically significant.

Ser1179 and Thr495 phosphorylation in MAs. eNOS phosphorylation at Ser1179 in MAs was evaluated with an antibovine Ser1179 antibody cross-reacting with phosphorylated (Ser1179) mouse eNOS. Phosphorylation of eNOS at Ser1179 was not observed in MA extracts from 2-mo-old WT or Gla-null mice. However, eNOS phosphorylation at Ser1179 was readily observed in MAs from 8-mo-old WT and Gla-knockout mice (Fig. 6A). A modest decrement in eNOS phosphorylation at Ser1179 was observed in MAs from Gla-null compared with WT mice (−21 ± 0.67%, F = 0.0343), consistent with the downregulation of eNOS activity (Fig. 6B). In contrast to this modest difference, phosphorylation of eNOS at Thr495 was markedly enhanced in MAs from aged Gla-null mice (Fig. 7A). A 16-fold increase in phosphorylation of eNOS at Thr495 was observed in MAs from 8-mo-old Gla-null compared with WT mice. The marked increase in Thr495 phosphorylation is consistent with a suppression of eNOS activity (Fig. 7B).
Increased reactive nitrogen species in MAs from Gla-null mice. Protein modification by nitration of tyrosine to 3-nitrotyrosine has been correlated with elevated oxidative stress and is specifically formed in the setting of eNOS uncoupling. The levels of protein-bound 3-nitrotyrosine in MAs from WT and Gla-null mice were measured using a specific antibody for protein-bound 3-nitrotyrosine. 3-Nitrotyrosine levels increased in mouse lines as a function of age (Fig. 8A). However, protein-bound 3-nitrotyrosine content was significantly higher (4-fold) in MAs from 8-mo-old mice. Data are expressed as percent change in lumen diameter relative to baseline. *P < 0.05, ***P < 0.001 vs. WT (by 2-way ANOVA followed by Bonferroni’s post hoc analysis).

DISCUSSION

Consistent with previous findings in other vascular beds (5, 22), we observed an age-dependent accumulation of Gb3 in the MAs of Gla-null, but not WT, mice. We also measured a significant reduction in the vasodilatory capacity of MA in Gla-deficient mice. These data suggest that the endothelial dysfunction associated with Gb3 accumulation is widespread within the vasculature and includes the gastrointestinal microvessels. Similar to the previous findings in aortic rings (17), an age- and endothelial cell-dependent dysfunction in the MA of Gla-null mice was observed, as manifest by a loss of ACh-stimulated vasodilation. The ability of SNP to restore the vasodilation was consistent with a primary defect in NO bioavailability. However, despite the profound nature of this defect, the spontaneous gastrointestinal phenotype was observed in Gla-null mice at any age. Surprisingly, the age of onset of the MA abnormality was earlier and the magnitude of the endothelial dysfunction appeared greater than in previously studied macrovessels, including the aorta and carotid arteries.

eNOS dysfunction may result from a decrease in NO bioavailability or enzyme uncoupling resulting in the formation of reactive nitrogen species. The active and functional form of eNOS is a dimer (19). We therefore measured the dimerization and expression of eNOS in MAs from Gla-deficient and WT mice. In the older mice, total eNOS expression and eNOS...
dimerization were decreased significantly, suggesting that uncoupling of eNOS in the MA may, in part, contribute to the endothelial dysfunction. However, the measurement of eNOS monomers was also lower in Gla-deficient mice, and no difference in the eNOS monomer-to-homodimer ratio was evident between WT and Gla-deficient mice at 2 or 8 mo of age. In previous studies examining the effect of age on endothelial function in C57BL6/J mice, a significant increase in the eNOS monomer-to-dimer ratio in 24- vs. 3-mo-old MAs was observed with no significant difference in total eNOS protein levels (26). Therefore, the reduction in eNOS protein and monomers in Gla-deficient mice does not appear to represent a simple acceleration of normal age-related changes. Rather, the changes likely represent a specific consequence of Gla deficiency, such as the accumulation of endothelial cell globo series glycosphingolipids.

NO production can lead to the formation of peroxynitrite (ONOO·), an NO-dependent nitrosative stress marker in the MA, was measured. 3-Nitrotyrosine was significantly elevated in MAs from Gla-knockout mice at 2 mo of age, and the levels of 3-nitrotyrosine were further elevated in the older Gla-null mice, consistent with the early onset and a progressive increase in eNOS uncoupling in MAs from Fabry mice.

To further characterize the altered eNOS regulation in Gla-deficient mice, posttranscriptional modifications to the eNOS protein were also evaluated. Phosphorylation of eNOS at Ser1179 and Thr495 was studied. In general, phosphorylation at Ser1179 activates eNOS activity, and phosphorylation at Thr495 inhibits eNOS catalytic activity (1, 24), and at least four protein kinases, Akt, PKA, PKC, and AMP-activated protein kinase, are known to target eNOS (15). Phosphorylation or dephosphorylation at Ser1179 and Thr495 potentially could be regulated reciprocally or independently by those kinases. PKC signaling is reported to result in simultaneous phosphorylation and dephosphorylation of eNOS at Ser1179 and Thr495, respectively (15). In addition, the phosphorylation state of Thr495 is inversely associated with eNOS activation and NO production mediated by ONOO·, the levels of 3-nitrotyrosine, an NO-dependent nitrosative stress marker in the MA, was measured. 3-Nitrotyrosine was significantly elevated in MAs from Gla-knockout mice at 2 mo of age, and the levels of 3-nitrotyrosine were further elevated in the older Gla-null mice, consistent with the early onset and a progressive increase in eNOS uncoupling in MAs from Fabry mice.

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Fig. 5. Endothelial nitric oxide synthase (eNOS) levels in MAs from 8-mo-old WT and Gla-null (KO) mice. Mouse MA lysates equivalent to 50 μg of total lysate protein were analyzed by Western blotting using a mouse anti-human eNOS antibody as an immunoprobe. A: representative immunoblot for comparison of eNOS expression in MAs from WT and Gla-knockout mice at 8 mo of age (top). The eNOS homodimer was only detected under nonreducing condition (lanes 1 and 2). Monomeric eNOS was probed under denaturing conditions using 1% 2-mercaptoethanol (lanes 3 and 4). β-Actin was also probed as an internal loading control (bottom). MW, molecular weight. B: quantification of monomeric and dimeric eNOS in MAs from 8-mo-old WT and Gla-null mice (n = 3 per group). Densitometric values are means ± SE. *P < 0.05.

Fig. 6. eNOS phosphorylation at Ser1179 in MAs from 8-mo-old mice. Total MA lysates equal to 80 μg of total lysate protein were subjected to a gradient SDS-PAGE (6–12%) separation and immunoblotting. Phosphorylation of eNOS at Ser1179 in MAs from WT and Gla-null (KO) mice was determined with a rabbit anti-bovine phosphorylated (Ser1179) eNOS (p-eNOS) antibody under nonreducing conditions. A: Western blots from 2 sets of MAs dissected from WT and Gla-null mice at 2 and 8 mo of age (top). β-Actin served as a loading control (bottom). B: combined densitometric data from 4 groups with 3 mice per group. Values are means ± SE. *P < 0.05.
Previous studies examining the function of Thr495 and Ser1179 mutated eNOS cells have suggested that the ratio of phosphorylated to dephosphorylated Thr 495 may act as an intrinsic switch for determining whether eNOS generates NO or superoxide anion (13). Therefore, we measured phosphorylation of eNOS at Thr495 in the MA to evaluate whether this site may be specifically altered in Gla-deficient mice.

eNOS phosphorylation at Thr495 was highly increased in the older Gla-deficient mice compared with age-matched WT mice, suggesting that phosphorylation at Thr495 may promote eNOS dysregulation in older Gla-null mice. Although we did not observe elevated phosphorylation at Thr495 at 2 mo of age, we did observe evidence of elevated 3-nitrotyrosine at this younger age. These data are consistent with the interpretation that the modest early-onset phenotype of endothelial dysfunction is linked to NO uncoupling through a mechanism that is independent of Thr495 phosphorylation. It is possible, therefore, that the more profound endothelial dysfunction at 8 mo is the result of the additional activation of the inhibitory eNOS Thr495 site.

In addition to alterations in NO, endothelium-derived hyperpolarizing factor is also an ACh-mediated factor and is at least as important as NO in terms of endothelium-dependent vasodilation in the microvasculature (4, 21). A recent study reported that Gb3 incubation partially inhibited ACh-mediated relaxation of mouse aortae (18). In that study, a significant decrease in Ca2⁺-activated K⁺ (KCa) channel current and mRNA and protein expression was observed in aortic endothelial cells from Gla-deficient mice and WT mice treated with Gb3. Also, pharmacological blockade of the small- and intermediate-conductance KCa channels has been demonstrated to increase superoxide formation and enhance eNOS phosphorylation at Thr495 without changes in the total eNOS protein levels (10). Thus Gb3 accumulation may lead to the earlier onset and profound endothelial dysfunction through direct effects on KCa channel activity and an endothelial plasma membrane defect, which results in impaired or altered signaling of multiple endothelium-dependent relaxing factors, including NO and endothelium-derived hyperpolarizing factor.

In conclusion, this study provides the first evidence of microvascular endothelial dysfunction in a mouse model of Fabry disease. The accumulation of Gb3 and decrease in eNOS bioavailability, along with an increase in 3-nitrotyrosine in the MA, are consistent with the phenotype previously observed in macrovessels. In addition, the reciprocal dysregulation of...
Ser\textsuperscript{179} and Thr\textsuperscript{495} in this vascular bed may also contribute to the profound arterial dysfunction. Whether eNOS dysfunction in the MA contributes to the gastrointestinal phenotype in humans with Fabry disease requires further investigation.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.J.K., L.S., J.A.S., and P.F.B. performed the experiments; J.J.K. and L.S. prepared the figures; J.J.K. and L.S. drafted the manuscript; J.J.K., L.S., J.L.P., J.A.S., and P.F.B. edited and revised the manuscript; J.J.K., L.S., J.L.P., J.A.S., and P.F.B. approved the final version of the manuscript.

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