Regional Cerebral Hyperperfusion and Nitric Oxide Pathway Dysregulation in Fabry Disease
Reversal by Enzyme Replacement Therapy

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Background—Fabry disease is an X-linked lysosomal deficiency of α-galactosidase A that results in cellular accumulation of galacto-conjugates such as globotriosylceramide, particularly in blood vessels. It is associated with early-onset stroke and kidney and heart failure.

Methods and Results—Using [15 O] H2O and PET, we found increased resting regional cerebral blood flow in Fabry disease without evidence of occlusive vasculopathy or cerebral hypoperfusion. Because nitric oxide is known to play an important role in vascular tone and reactivity, we studied plasma nitrate, nitrite, and low-molecular-weight S-nitrosothiol levels by chemiluminescence. Skin biopsy specimens and archived brain tissue were also examined immunohistochemically for nitrotyrosine. Plasma nitrate, nitrite, and low-molecular-weight S-nitrosothiol were in the normal range; however, enhanced nitrotyrosine staining was observed in dermal and cerebral blood vessels. After a double-blind, placebo-controlled trial of α-galactosidase A therapy, the resting regional cerebral blood flow in the treated group was significantly reduced, with a notable decrease of nitrotyrosine staining in dermal blood vessels.

Conclusions—These findings suggest a chronic alteration of the nitric oxide pathway in Fabry disease, with critical protein nitration that is reversible with enzyme replacement therapy. (Circulation. 2001;104:1506-1512.)

Key Words: cerebrovascular disorders ■ nitric oxide ■ tomography ■ regional blood flow ■ cerebrovascular circulation

Fabry disease is an X-linked disorder resulting from mutation of the α-galactosidase A gene at Xq22.1 The gene defect results in very low specific activity of the lysosomal enzyme α-galactosidase A, with impaired metabolism of terminal α-D-galactosyl moieties.2 This abnormality impairs the conversion of globotriosylceramide (Gb₃) to lactosylceramide with intralysosomal accumulation of glycosphingolipids, particularly Gb₃. Systemic glycosphingolipid accumulation occurs with a predilection for vascular endothelial and smooth muscle cells, myocardium, renal epithelium, and the central nervous system (CNS). In the CNS, diffuse storage occurs in the cerebral vasculature, with more localized involvement of central neurons together with the dorsal root and autonomic ganglia in the peripheral nervous system.2 In the skin, dermal vessels undergo dilation and proliferation to form angiokeratoma. Hemizygous patients have a median survival of 41 years,2 with death secondary to kidney failure, myocardial infarction, and cerebrovascular disease. The cerebrovascular manifestations consist of large-vessel ectasia, large-vessel occlusive disease, and small-vessel occlusive disease. The vascular diathesis is reported to have a vertebrobasilar circulation distribution,3,4 although the reason for this is unclear.

The burden of cerebrovascular disease, best observed in fluid-attenuated inversion recovery (FLAIR) or T2-weighted MR sequences, increases with age, with progressive radiologic evidence of disease after the age of 30 years.5 Although it is thought that the progressive deposition of Gb₃ within the vascular endothelial cells results in cerebral hypoperfusion and eventual occlusive disease, this has not been demonstrated in vivo.6 To test for subclinical cerebral hypoperfusion, we examined the regional cerebral blood flow (rCBF) in Fabry disease by using [15 O] H2O and PET. We also hypothesized that the regional cerebral perfusion abnormality will be
Corrected by enzyme replacement therapy. The rCBF abnormalities that we found in Fabry disease led us to look for evidence of nitric oxide (NO) pathway dysfunction.26 We examined levels of plasma NO metabolites, together with dermal and cerebral vascular tyrosine nitration.

Methods

Patients and Control Subjects
Twenty-six consecutive male hemizygous patients with Fabry disease and neuropathic pain (age range, 19 to 47 years) and 10 consecutive healthy volunteer subjects (age range, 21 to 48 years) were enrolled in the PET study. Only 1 enrolled patient had a history of stroke ~10 years before the current study. The Institutional Review Board of the National Institute of Neurological Disorders and Stroke and the National Institutes of Health Radiation Safety Committee approved the studies. All patients and control subjects gave their written informed consent. All patients and control subjects had the following cerebral MRI studies: T1- and T2-spoiled gradient recalled acquisition, FLAIR, axial T2-, and diffusion-weighted imaging. In 3 study patients, a mild amount of white matter, high-signal abnormality was found on FLAIR imaging; in another 3 patients, between 1 and 3 discrete lesions were noted. The double-blind, placebo-controlled, randomized trial of enzyme replacement therapy (ERT) consisted of intravenous infusions of α-galactosidase A every 2 weeks (Trans-karyotic Therapies, Inc), with 14 patients receiving ERT and 12 receiving placebo for 6 months.

PET Procedure With [15O] H2O
Positron emission tomography was performed with a Scanditronix PC2048–15B tomograph, which collects 15 simultaneous slices with an in-plane and axial resolution of 6.0 mm. All participants had arterial line placement, allowing measurement of the H2O15O input function and arterial blood gases. All participant scans were performed before and after enzyme replacement therapy or placebo while control subjects were studied on one occasion only. At least one axial slice was obtained within the posterior fossa as determined by the initial transmission scan. Each study commenced with a sham scan to acclimatize the subject to the scanning procedure and counteract procedure-related anxiety. All participants then underwent replicate scans, with each scan commenced on arrival of radioactivity in the head after intravenous injection of 35 mCi of [15O] H2O. A total 12, 10-second scans and 4, 30-second dynamic scan frames were acquired after tracer injection and reconstructed with a Hanning filter together with correction for attenuation, normalization, scatter, and dead-time. The arterial input function was measured with an automated blood counting system, with blood drawn from the arterial line and the time shift between blood and brain data determined. Parametric CBF images were constructed from a pixel-by-pixel, least-square fit for blood flow and tracer partition coefficient.9 The units of the final CBF parametric images are in milliliters per minute per 100 g of brain tissue. In the Fabry patients, all PET studies were repeated at the end of the 6-month, randomized, double-blind, placebo-controlled trial of α-galactosidase A; the control group studies were interspersed over the total study period.

Data Analysis
Both the patient and control CBF scans were coregistered to MR images of the brain so that regional CBF comparisons could be made between the patient and control group. The MRI template created by registering 27 scans from the same individual (T1-weighted gradient-echo acquisition with TR/TE/FA = 18 ms/10 ms/30°) into the same stereotaxic space10 provided a high-resolution, low signal-to-noise image set. Non-CNS tissue was edited out by hand with custom-written OpenGL code on a Silicon Graphics workstation. Before spatial registration, the PET and MRI data were resampled to 256×256×300, 0.9375-mm isotropic voxels. The replicated resting CBF images were averaged. Spatial registration between the PET images and the T1 MRI template was performed with the use of SPM.11 Statistical parametric t-maps (SPM{t}) were calculated with the use of SPM on the spatially registered PET images by obtaining a global set-level statistic of P<0.05 after setting the voxel threshold at P<0.05 and the cluster extent at ≥1024. The cluster extent threshold represents ~1 cm3 of brain tissue. Data from the pretreatment Fabry group were compared with the control group. In the enzyme replacement trial, the pretest images were subtracted from the posttest images for each patient, followed by comparison of the difference images between treatment and placebo groups.

Global CBF Measurements
The parametric PET images were averaged on a voxel-by-voxel basis after setting a voxel threshold CBF of 10 mL/min per 100 g of tissue. All global CBF comparisons were made with the use of a t test with a Satterthwaite correction for unequal variance.

White Matter–Gray Matter Distribution of CBF
The T1 MRI template was further segmented into gray and white matter images by using the technique of optimal thresholding. The shape of the distribution was represented by gaussian curves of varying amplitude (A), mean values (μ), and standard deviations (σ). The values for A, μ, and σ were calculated by means of the Marquardt-Levenberg nonlinear optimization. The optimal threshold was then determined by a Golden Section search routine.12 The T1 MRI template was then converted into white and gray matter images. These images were combined with the SPM{t} maps using a logical AND filter, with the percentage of the total white or gray matter pixel count used to express the final result.

Processing of Blood and Ozone-Based Chemiluminescent Determination of Serum Nitrate, Nitrite, and Low-Molecular-Weight S-Nitrosothiol
Peripheral venous blood samples were drawn from a further set of 8 control subjects and 9 enzyme treatment–naïve patients after a 3-day low nitrate diet. They were collected in EDTA tubes and centrifuged at 750g for 5 minutes. Blood samples were collected with and without 8 mmol/L N-ethyl-maleimide to prevent breakdown of low-molecular-weight S-nitrosothiol (LMW-SNO).13 Aliquots of plasma were stored at −70°C before nitrate, nitrite, and LMW-SNO determination. On thawing, the plasma samples were filtered through prewashed 10 000 molecular weight cutoff filtration units and injected with different reductants in line with 1N NaOH and the Sievers Model 280 NO analyzer. Nitrate was assayed by reduction in vanadium(III) at 90°C; nitrite was assayed by reduction in acidified KI (7 mL of glacial acetic acid/2 mL of distilled water/50 mg of KI). S-nitrothiols from plasma treated with 8 mmol/L N-ethyl-maleimide (final plasma concentration) were measured by using the Cu2+/L-cysteine chemiluminescent assay, which is specific for S-nitrosothiol detection. A linear sensitivity was obtained in all assays >1.0 pmol of nitrate, nitrite, and SNO-glutathione (r2 =0.99, P<0.001).

Immunohistochemistry of eNOS, iNOS, nNOS, and Nitrotyrosine
Polyclonal antiserum used in this study were rabbit anti-PGP 9.5 (Biogenesis Inc), rabbit anti-laminin (L9393, Sigma Chemical), and rabbit anti-nNOS (N-20, Santa Cruz Biotechnology). Monoclonal antibodies used were eNOS (clone 3, Transduction Laboratories), iNOS (N32030, Transduction Laboratories), nitrotyrosine (32 to 1900, Zymed Laboratories), and nNOS (clone 2A3; NeoMarkers). Tissue sections were counterstained with Alexa 488-goot anti-rabbit antibody (Molecular Probes Inc) to visualize polyclonal staining and Cy3-goot anti-mouse antibody (Jackson ImmunoResearch) to visualize monoclonal staining. Three-millimeter skin biopsy specimens were removed from the dorsolateral thigh. All tissue was processed for immunohistochemistry as previously described.14 Sections were stained free-floating by overnight incubation with a given antiserum and monoclonal antibody diluted in Tris-buffered saline (TBS) containing 0.5% Triton X-100 and 3% normal goat serum (TBS stain
buffer) at 4°C. Sections were washed in TBS with 0.1% Triton X-100 (TBS wash buffer), then counterstained with Alexa 488 and Cy3 secondary antibodies in TBS stain buffer (4 hours at room temperature or overnight at 4°C). After removal of the secondary antibodies in TBS wash buffer, the sections were mounted in Vectashield (Vector Laboratories) on slides and examined by epifluorescence (Axioscope, Carl Zeiss) and laser confocal microscopy (MRC 1024, Bio-Rad). Biopsies from 7 randomly chosen patients, 4 in the ERT group and 3 in the placebo group, were examined in a paired fashion before and after the double-blind, placebo-controlled trial. All staining was performed concurrently with control tissue from healthy volunteers. Nitrotyrosine immunohistochemistry was performed on Fabry and control brain tissue (obtained from the Brain and Tissue Bank, University of Miami, Fla).

**Results**

Magnetic resonance imaging, performed on all subjects with and without Fabry disease, indicated radiologically similar groups. $P_{O_2}$, $P_{CO_2}$, and arterial pH during the PET CBF studies were not significantly different for any study group. Resting global CBF was not significantly different between patients and control subjects (CBF Fabry group, $n=26$, $42.0\pm4.8$; CBF control group, $n=10$, $39.1\pm4.8$ mL/min per 100 g of tissue). Analysis of the resting rCBF in the Fabry group showed regions of significantly increased blood flow in the brain stem, cerebellum, and bilateral temporal, posterior occipital, and inferior frontal regions (set-level statistic, $P=0.014$; voxel height threshold, $P=0.034$; and extent threshold, 1024; Figure 1). The rCBF was not significantly lower in the patient group compared with the control group at any set-level statistic. At rest, the significantly elevated rCBF voxels in the Fabry patients comprised 8.3% of the total white matter voxels and 11.0% of the total gray matter voxels. This finding indicates that the abnormality in resting rCBF in Fabry disease is relatively equally distributed between white and gray matter, suggesting a global disease process rather than a selective vulnerability, although as previously reported, the disease predominantly affected the vertebrobasilar circulation.3,4

After enzyme replacement, the resting global CBF fell significantly in the treated group, whereas in the placebo group it increased (paired CBF change ERT group, $-3.48\pm4.57$ mL/min per 100 g of tissue; paired CBF change placebo group, $1.25\pm5.11$ mL/min per 100 g of tissue, $P=0.0258$). A significant decrease in rCBF also occurred in the treatment group before and after ERT compared with the placebo group (set-level statistic, $P=0.019$; voxel height threshold, $P=0.034$; extent threshold, 1024; Figure 2), whereas there were no areas of significantly decreased rCBF in the placebo group compared with the enzyme-treated group.

Plasma nitrate (Fabry, $54.95\pm20.02$ μmol/L, $n=9$; control, $71.46\pm38.53$ μmol/L, $n=8$; $P=0.301$), nitrite (Fabry, $821\pm253$ nmol/L, $n=8$; control, $860\pm311$ nmol/L, $n=8$; $P=0.787$), and LMW-SNO (undetectable) were not significantly different in Fabry disease compared with control subjects after a low-nitrate diet. Tissue immunohistochemistry for nitrotyrosine performed on Fabry skin demonstrated excess dermal vascular nitrotyrosine staining in all untreated Fabry patients compared with the control group (Figure 3). There was notable decrease in nitrotyrosine staining after 6 months of enzyme replacement therapy ($n=4$). No difference in nitrotyrosine staining was seen in the placebo group before
and after enzyme replacement (n=3). Furthermore, no difference was found in dermal vascular eNOS (Figure 4), iNOS, and nNOS staining (data not shown) between control subjects and Fabry patients before or after enzyme therapy. Excess nitrotyrosine staining was seen in Fabry brain vessels compared with control brain vessels (Figure 5). The staining density was particularly high in the intimal region. Staining for eNOS had similar intensity in Fabry brain and control brain tissue (not shown).

Discussion

Our study demonstrates reversal of functional cerebral blood flow abnormalities together with tissue abnormalities in tyrosine nitration after ERT in $\alpha$-galactosidase A deficiency. This is consistent with the response to treatment found in other organ systems. The finding of elevated resting rCBF in Fabry disease prompted the question of whether the underlying glyco-conjugate disorder results in excessive neuronal activity increasing neuronal–blood flow coupling and causing cerebral hyperperfusion. Such metabolic abnormalities resulting in elevation of regional cerebral glucose metabolism can be seen in other lysosomal storage disease such as Salla’s disease, a type of lysosomal storage disorder involving sialic acid metabolism. A finding of elevated cerebral glucose utilization through the use of FDG and PET would indicate the likelihood of neurally driven rCBF coupling. If, however, the rCBF and regional cerebral glucose metabolism were found to be uncorrelated, then a primary cerebrovascular abnormality would be more likely. Concurrent work is underway to clarify the above finding. The rCBF elevation in the placebo group is consistent with disease progression over the 6-month trial period.

Other data indicating abnormal vascular function in Fabry disease was found by using forearm venous plethysmography. These data indicate hyperreactivity of the forearm vascular bed in Fabry disease in response to intra-arterial acetylcholine, which was only partially inhibited by N-monomethyl-L-arginine, suggesting an abnormality in the nonnitric oxide pathway, possibly endothelial hyperpolarizing factor, controlling arterial vessel tone. These results were subsequently corroborated by an independent measurement of the forearm vessel flow. Recent evidence suggests that endothelial hyperpolarizing factor might be hydrogen peroxide.

Because of the known involvement of NO in basal cerebrovascular tone, we examined patients with Fabry disease further for abnormalities of the NO pathway. A normal plasma nitrate level suggests normal systemic production of NO. Nitrate is produced by the reaction of NO with oxyhemoglobin (HbFe$^{6+}$O$_2$), resulting in methemoglobin (HbFe$^{3+}$) and nitrate (NO$_3^-$) formation. This pathway is considered the major inactivation pathway of vascular NO. Nitrite (NO$_2^-$) and LMW-SNO formation occurs when NO reacts with O$_2$, resulting in the intermediate species dinitrogen trioxide (N$_2$O$_3$). These species therefore reflect endothelial NO production in Fabry disease. Although the level of plasma nitrate was not significantly different from that in control subjects, the mean value was decreased in Fabry disease. This result could be due to a lack of statistical power. A depressed plasma nitrate level might indicate altered NO regulation because local tissue NO could be increased and diverted into...
critical protein nitration as the result of the underlying metabolic abnormality in Fabry disease. This would especially occur if excess superoxide (O$_2^-$) were also present.

α-Galactosidase A deficiency may represent a state of excessive tissue NO production, together with NO/O$_2^-$ imbalance. NO may then react with O$_2^-$ to form peroxynitrite (ONOO$^-$), resulting in “oxidative stress,” in which critical enzyme and protein amino acid moieties are oxidized, leading to dysfunctional cellular activity and sometimes death.22 At a cellular level, NO competes with superoxide dismutase in

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Figure 3. Dermal nitrotyrosine (a, c, e) and laminin (b, d, f) immunohistochemical localization in control skin and in Fabry patients before and after ERT. a, Control skin showing nitrotyrosine staining (rhodamine filter, ×10 magnification) and laminin staining; b, fluorescent filter, ×10 magnification of dermal vessels (arrows) and stratum basalis (same section). c and d, Nitrotyrosine staining before ERT. Significant perivascular staining (arrows) after ERT dermal vascular staining is no longer apparent (e, f).

Figure 4. eNOS immunoreactivity in control and Fabry patient skin biopsy specimens. a, Normal control eNOS staining; b, same section double-labeled with anti-laminin antibody. c and d, Dermal eNOS staining in Fabry disease before and after placebo; e and f, dermal eNOS staining before and after ERT. No difference in dermal or vascular eNOS staining was found. All panels are in the same magnification. Scale bar, 100 μm.
scavenging $\text{O}_2^\cdot$. Increased tissue levels of NO with formation of ONOO$^-$ would be expected to result in cellular peroxidation with excess peroxynitrite and nitrotyrosine formation.$^{23}$ Such a process would be more pronounced in the presence of excess $\text{O}_2^\cdot$. Although reactive nitrogen species–mediated stress has been implicated in the pathogenesis of neurological diseases such as amyotrophic lateral sclerosis,$^{24}$ we show a direct association with a functional abnormality in Fabry disease.

The decrease in the dermal vascular nitrotyrosine staining after 6 months of ERT is consistent with both a treatment effect and reduced NO-induced “oxidative stress.” The reason for the persistent nitrotyrosine staining despite ERT in the epidermis may be due to decreased accessibility to enzyme. It is probable that the same pathogenic mechanism is involved in the development of the dermal vasculopathy of Fabry disease as in the cerebral vasculature, a view strengthened by similar nitrotyrosine staining in both vessel beds. It has been shown that peroxynitrite can induce cerebral vasodilation with subsequent resistance to vasoconstriction by humoral mediators.$^{25}$ Such an effect could account for the hyperperfused cerebral circulation in Fabry disease, together with the skin findings. Skin immunohistochemistry also showed no difference in the localization of eNOS, nNOS, and iNOS staining between control subjects and in patients with Fabry disease before and after ERT. This finding together with the increased tissue nitration suggests that deficiency of $\alpha$-galactosidase A results in an abnormality of NOS function at a membrane or cytoplasmic level, leading to excessive NOS activity. A key candidate for this abnormality is elevation in the level of cytoplasmic $\text{Ca}^{2+}$. Other mechanisms such as excessive $\text{O}_2^\cdot$ generation remain possible, and cerebral arteriolar vasodilation secondary to $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$ has been described.$^{27}$

The relation between the increased risk of stroke in Fabry disease and the elevated rCBF is unclear. It is possible that the increased rCBF contributes to endothelial dysfunction and vessel wall dilation, resulting in a procoagulant and abnormal flow state increasing the incidence of emboli or thrombosis. Further elucidation of the cellular mechanisms underlying the single gene defect in Fabry disease may hold insights into the more complex multifactorial pathogenesis of stroke in general. For example, it is possible that cerebrovascular dysfunction is present in asymptomatic patients prone to develop strokes from other causes such as atherosclerosis. If so, identifying vascular functional abnormalities through the use of PET or MR techniques may facilitate the assessment of preventive therapies.

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**References**

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