Sex differences of urinary and kidney globotriaosylceramide and lyso-globotriaosylceramide in Fabry mice

Brandon Durant,†, * Sabrina Forni,†, * Lawrence Sweetman, * Nastry Brignol,† Xing-Li Meng, * Elfrida R. Benjamin,† Raphael Schiffmann,‡,* and Jin-Song Shen *

Institute of Metabolic Disease, * Baylor Research Institute, Dallas, TX; and Amicus Therapeutics, Inc., ‡ Cranbury, NJ

Abstract The aim of our study was to measure globotriaosylceramide (Gb₃) and lyso-Gb₃ levels by tandem mass spectrometry in the urine and kidney in Fabry (gla knockout) mice and wild-type controls. We found that urine Gb₃ of male and female Fabry mice was higher than wild-type mice of the same sex but also significantly higher in male mice compared with females of the same genotype. In kidney tissue, sex and genotype-dependent differences in Gb₃ levels paralleled those in the urine. Isoforms C16, C22:1, and C24OHA were particularly higher in males compared with females in both wild-type and Fabry mice. Similarly, kidney lyso-Gb₃ concentrations were significantly higher in 12-month-old male Fabry mice than in their homozygous female counterparts. However, lyso-Gb₃ was undetectable in wild-type mice of both sexes. α-Galactosidase A activity and mRNA levels in kidney were significantly lower in male wild-type mice compared with female mice. This study shows the sex differences in kidney and urine Gb₃ and kidney lyso-Gb₃ levels in both wild-type and Fabry mice, and it suggests that these male-female differences should be taken into consideration when using murine models for Fabry disease.—Durant, B., S. Forni, L. Sweetman, N. Brignol, X-L. Meng, E. R. Benjamin, R. Schiffmann, and J-S. Shen. Sex differences of urinary and kidney globotriaosylceramide and lyso-globotriaosylceramide in Fabry mice. J. Lipid Res. 52: 1742–1746.

Supplementary key words glycosphingolipids • testosterone • urine • mass spectrometry

Sex differences of urinary and kidney globotriaosylceramide and lyso-globotriaosylceramide in Fabry mice have been described (1). This model has been used for preclinical studies of new therapeutics for Fabry disease, such as enzyme replacement therapy, and for studies of Fabry disease mechanisms with outcome measurements based predominantly on measurement of Gb₃ levels (11–13). However, the concentrations of urinary Gb₃ in the Fabry mouse model have not been described and little attention has been paid to the male versus female murine Gb₃ levels in wild-type and knockout Fabry mice (14–16). Recently, these mice have been shown to accumulate deacylated Gb₃, globotriaosylphosphoglycerine (lyso-Gb₃) (17). In humans, lyso-Gb₃ was shown to be elevated in plasma and urine of Fabry patients and thus represents a potential new biomarker for Fabry disease (18). In the present study, we evaluated the concentrations of urinary and kidney Gb₃ levels, as well as kidney lyso-Gb₃ levels, in male and female Fabry mice, and we compared these to their respective wild-type controls.

MATERIALS AND METHODS

Mice

Hemizygous male and homozygous female gla knockout mice (referred to as Fabry mice; C57Bl6/SVJ129) (7) and wild-type control mice of the same genetic background were used.
**Urine collection**

Five to eight urine samples (collected separately from each mouse) were collected from each mouse group for analysis. All specimens were collected over a 24 h duration using polycarbonate metabolic cages (model 5600M021, Tecniplast, Montreal, Quebec, Canada). Urine samples were collected at room temperature and stored at -80°C until analysis by mass spectrometry.

**Analysis of urinary Gb3**

Ten microliters of 250 ng/ml C17-Gb3 (Matreya, LLC, Pleasant Gap, PA) in methanol were added to 100 µl urine as the internal standard. Each sample was extracted with 1 ml of methanol; 5 µl of extract were injected in full loop mode in a UPLC-MS/MS system (Premier XE; Waters Corp., Milford, MA) equipped with ESI in positive ion mode (capillary 3.5 kV, cone 50 V, source 100°C, desolvation 400°C, cone gas 50 l/h, desolvation gas, 500 l/h). Eight isoforms were separated with a C18 Acquity BEH 100 × 1 mm, 1.7 µm column with a methanol/water and 0.1% formic acid step gradient. The following transitions were monitored in MRM mode: m/z 1046→884 C16, 1074→912 C18, 1102→940 C20, 1128→966 C22:1, 1130→968 C22, 1156→994 C24:1, 1158→996 C24, 1174→1012 C24OH. The ratio of the peak area sum of these eight transitions to that of the internal standard was determined. This ratio was used to calculate the total Gb3 concentration based on linear regression of the Gb3 reference standard calibration curve generated by standard addition in urine (19).

**Analysis of kidney Gb3 and lyso-Gb3**

Kidney tissue was harvested from 5- and 12-month-old mice. Gb3 concentration in tissue was determined as described below.

**Tissue Gb3 quantification.** Liquid-liquid extraction with methyl tert-butyl ether followed by saponification was performed on tissue homogenate corresponding to 200 mg of total protein. Each sample was reconstituted in water/methanol 20/80 (v/v), and 5 µl were injected into a UPLC-Xevo tandem mass spectrometry system (Waters Corp.). Separation was performed on a C18 Acquity BEH 100 × 1 mm, 1.7 µm column with a methanol/water and 0.1% formic acid gradient. The following transitions were monitored in MRM mode: m/z 1046→884 C16, 1074→912 C18, 1102→940 C20, 1128→966 C22:1, 1130→968 C22, 1156→994 C24:1, 1158→996 C24, 1174→1012 C24OH. A Gb3 reference standard (Matreya) calibration curve in matching matrix prepared with the standard addition method was run in each assay for calculation of the concentration of total Gb3 in each sample. Gb3 was calculated as the sum of the concentrations of each monitored isoform. The concentration of each isoform was determined by linear regression with the internal standard. Total Gb3 was calculated using the software Targetlynx (Waters). Results were normalized to the amount of total protein (in milligrams) in each sample.

**Tissue lyso-Gb3 quantification.** Kidney tissue samples (up to 50 mg each) were prepared as previously described (20). Lyso-Gb3 was assayed as previously described (18).

**α-Galactosidase A assay**

α-galactosidase A activity in kidney homogenates was determined by a fluorimetric method as described previously (21).

**Quantitative RT-PCR**

Quantitative real time RT-PCR was performed as previously described (3). Predesigned TaqMan probe and primers for mouse gla were purchased from Applied Biosystems, Inc. (Foster City, CA). 18S rRNA was used as internal control and detected by TaqMan probe and primers (Applied Biosystems, Inc.).

**Statistical analysis**

For relative mRNA levels, statistical significance was determined by Mann-Whitney test. Two-tailed Student’s t-test was used for analysis of other data. The data were presented as the mean ± SE.

**RESULTS**

Consistent with previous observations (14–16), urinary Gb3 levels were significantly higher in adult wild-type male mice compared with wild-type female mice (Fig. 1A). Urinary Gb3 levels were lower in prepubertal (three-week-old) wild-type males [399 ± 31 ng/ml (n = 3)] compared with adult wild-type males, but they were similar to the urinary Gb3 levels measured in one prepubertal female (255 ng/ml).

Similarly, urinary Gb3 was significantly higher in Fabry male mice compared with homozygous Fabry female mice (Fig. 1A). The urinary Gb3 level in Fabry homozygous females was significantly higher than in wild-type females.

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**Fig. 1.** Urinary and kidney Gb3 and lyso-Gb3 contents in mouse kidneys. A: Urine Gb3 levels at 5 months of age. Urine Gb3 levels were significantly higher in males compared with females in both wild-type and Fabry mice (n = 5-6). The increment of Gb3 levels in Fabry mice compared with wild-type mice was statistically significant in females but not in males.*P < 0.0001, wild-type female versus Fabry female. B: Kidney Gb3 levels at 5 months of age for wild-type and Fabry and Fabry at 12 months of age. Kidney Gb3 levels were markedly higher in males compared with females in both wild-type and Fabry mice. The increment of Gb3 levels in Fabry mice compared with wild-type mice was statistically significant in females but not in males.*P < 0.01, wild-type female versus Fabry female at 5 months of age (n = 3); **P < 0.03, Fabry male versus Fabry female at 12 months of age (n = 8).
should be taken into consideration when using murine models for Fabry disease.

Our finding of markedly elevated urinary and kidney Gb3 in mature male wild-type mice compared with females confirms previous observations (14–16). This is a general murine phenomenon, as it was also present in B6CBA mice and other strains (15). In humans, however, no difference in urinary Gb3 levels was found between male and female patients (19). The male-female difference in Gb3 (and other related glycosphingolipids) level in mouse kidney is thought to be due to increased synthesis caused by testosterone in male mice (15, 16). In this study, we found significantly lower kidney Gb3 activity in male mice compared with females (Fig. 1A). Although the urine Gb3 level in Fabry hemizygous male mice was higher than in wild-type controls, this difference was not significant (Fig. 1A).

A considerable amount of glycosphingolipid in mouse urine is excreted from the kidney, and glycosphingolipid content of urine is correlated with that in kidney tissues (14). We measured Gb3 levels in mouse kidneys and found markedly elevated Gb3 levels in males compared with females in both wild-type and Fabry mice (Fig. 1B). Fabry female mice had significantly higher kidney Gb3 levels compared with age-matched, wild-type females (Fig. 1B). In parallel to observations in urine Gb3, the increment of kidney Gb3 in Fabry male mice compared with wild-type male mice at five months of age was not statistically significant (Fig. 1B). We also measured lyso-Gb3. It was undetectable in male and female wild-type mouse kidney. However, lyso-Gb3 was measurable in kidney of Fabry KO mice where it was found to be significantly higher in 12-month-old male Fabry mice compared with homozygous female Fabry mice (Fig. 1C).

To determine whether there is any specific isoform that predominantly contributes to sex differences in kidney Gb3 levels, the concentration of individual isoforms of Gb3 were compared between males and females (Fig. 2A, B). In both wild-type and Fabry mice, isoforms C16, C22:1, and C24OHA were relatively more predominant in males compared with females (Fig. 2A, B). The concentration of each isoform was also compared between Fabry and wild-type mice (Fig. 2A, B). The concentration of each isoform was also compared between Fabry and wild-type mice (Fig. 2C). The fold increase in Fabry mouse kidney Gb3 concentration compared with wild-type was greater in females than in males for all Gb3 isoforms (Fig. 2C). This increase ranged from a 2-fold (C24OHA) to 10-fold (C24_B) greater concentration of Gb3 in Fabry males compared with wild-type males, and from a 15-fold (C24OHB) to 58-fold (C22:1) greater concentration of Gb3 in Fabry females compared with wild-type females.

Testosterone increases synthesis and excretion of glycosphingolipids in mouse kidney (14–16). Thus, different levels of testosterone in adult male and female mice can cause sex differences in kidney and urine Gb3. On the other hand, testosterone increases the synthesis of some lysosomal enzymes such as β-glucuronidase and β-galactosidase in murine kidney (22, 23). To determine whether the lysosomal hydrolysis of Gb3 contributes to sex differences in kidney Gb3, we measured α-galactosidase A activity in kidney tissues. We found significantly lower α-galactosidase A activity in wild-type males compared with wild-type females (Fig. 3A). To determine whether this lower enzyme activity is due to lower expression level of the enzyme, mRNA level of α-galactosidase A in kidney tissues was measured by quantitative RT-PCR. The results showed significantly lower mRNA level in wild-type males compared with wild-type females (Fig. 3B).

DISCUSSION

This study shows the sex differences in kidney and urine Gb3 levels and kidney lyso-Gb3 levels in both wild-type and Fabry mice and suggests that these male-female differences should be taken into consideration when using murine models for Fabry disease.

Our finding of markedly elevated urinary and kidney Gb3 in mature male wild-type mice compared with females confirms previous observations (14–16). This is a general murine phenomenon, as it was also present in B6CBA mice and other strains (15). In humans, however, no difference in urinary Gb3 levels was found between male and female patients (19). The male-female difference in Gb3 (and other related glycosphingolipids) level in mouse kidney is thought to be due to increased synthesis caused by testosterone in male mice (15, 16). In this study, we found significantly lower kidney α-galactosidase A activity in male mice compared with females. This suggests that the lower
The deficient enzyme. Besides the sex difference in total due to a relatively more rapid Gb\(_3\) accumulation in the compared with 5 months of age) (Fig. 2B). This is probably there is an apparent decrement of male/female ratio of presence of sex differences in kidney and urine Gb\(_3\) and the kidney (7, 8). However, our study demonstrated the completely by intravenously infused alpha-galactosidase A or other isoforms may be testosterone inducible. Ioannou et al. reported that one of the two Gb\(_3\) bands of Fabry mouse kidney extract in thin layer chromatography does not respond to administered enzyme (9). It is possible that the three isoforms described above may be the major components of the enzyme-resistant Gb\(_3\) band. In addition, there is an apparent decrement of male/female ratio of most Gb species in older Fabry mice (12 months compared with 5 months of age) (Fig. 2B). This is probably due to a relatively more rapid Gb\(_3\) accumulation in the female Fabry mice (Fig. 1B).

Despite the significant accumulation of Gb\(_3\) and lyso-Gb\(_3\) in kidney of Fabry mice shown in this study and others (17) and the pathological changes in kidney tissues (8, 10), there are no obvious clinical abnormalities such as proteinuria and renal failure, which are common in Fabry patients, reported in Fabry mice. A systematic characterization of kidney phenotype in Fabry mice including potential sex differences in these phenotypes is in progress.

The proportion of testosterone-induced Gb\(_3\) to total Gb\(_3\), its chemical properties, and its biological role in Fabry mouse kidney are not clear. However, experimentally, this form of Gb\(_3\) complicates the relationship between alpha-galactosidase A activity and Gb\(_3\) level in kidney of male Fabry mice. Our study suggests that if one looks at Gb\(_3\) levels in kidney or urine as an outcome parameter for preclinical proof-of-concept studies evaluating new therapeutic approaches, female homozygous mice should be the better model to use because it is more straightforward and may give more sensitive and clearer results.

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