Blocking hyperactive androgen receptor signaling ameliorates cardiac and renal hypertrophy in Fabry mice

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Abstract

Fabry disease is caused by deficient activity of lysosomal enzyme α-galactosidase A. The enzyme deficiency results in intracellular accumulation of glycosphingolipids, leading to a variety of clinical manifestations including hypertrophic cardiomyopathy and renal insufficiency. The mechanism through which glycosphingolipid accumulation causes these manifestations remains unclear. Current treatment, especially when initiated at later stage of the disease, does not produce completely satisfactory results. Elucidation of the pathogenesis of Fabry disease is therefore crucial to developing new treatments. We found increased activity of androgen receptor (AR) signaling in Fabry disease. We subsequently also found that blockade of AR signaling either through castration or AR-antagonist prevented and reversed cardiac and kidney hypertrophic phenotype in a mouse model of Fabry disease. Our findings implicate abnormal AR pathway in the pathogenesis of Fabry disease and suggest blocking AR signaling as a novel therapeutic approach.

Introduction

Fabry disease is an X-linked lysosomal storage disorder. It is caused by deficient activity of lysosomal enzyme α-galactosidase A (α-gal A) (1). Deficiency of α-gal A results in an inability of the cells to catabolize glycosphingolipids (GSL) with terminal α-D-galactosyl residues. These GSLs, particularly globotriaosylceramide (Gb3), accumulate progressively in virtually all organs and systems, resulting in progressive multisystemic damage (2,3).

Fabry disease exhibits a variety of clinical manifestations, of which cardiovascular and renal complications are the most life threatening. Left ventricular hypertrophy is a key feature in Fabry disease. In most cases, the hypertrophy is concentric, leading to diastolic dysfunction and fibrosis. Systolic dysfunction occurs later in the course of the disease (4). Renal manifestations occur in most Fabry patients. Kidneys of Fabry patients are enlarged in the third decade of life, followed by gradually decreasing size after the fourth decade (5). Proteinuria and decreased glomerular filtration rate progress over time, leading to end-stage renal disease (2,3). Although male patients usually have the most severe form of the disease, heterozygous female patients may also be affected, and disease severity depends also on the degree of inactivation of the affected X-chromosome in each organ (6).

Enzyme replacement therapy (ERT) is currently the only specific treatment available for this disease. ERT is effective in reducing GSL accumulation in tissues and appears to slow
progression of the disease (7). Recent studies suggested that ERT is more efficacious when initiated at earlier disease stages (8,9). In general, however, the clinical effect of ERT in Fabry disease is insufficient to stop or reverse most disease processes when initiated in adults. There is therefore significant need to develop novel therapeutic approaches that will be based on extending our understanding of Fabry disease beyond the primary enzyme deficiency.

The mechanisms by which accumulation of GSL leads to the clinicopathological manifestations of Fabry disease are poorly understood. Previous studies suggested that oxidative stress and endothelial nitric oxide synthase dysfunction are involved in the vasculopathy of this disease (10,11). Recently, it has been found that the level of deacylated Gb3 (lyso-Gb3) is markedly elevated in Fabry patients’ plasma and that lyso-Gb3 may contribute to vascular remodeling through proliferation of smooth muscle cells (12). In addition, the lysosomal-autophagy system was found to be abnormal in Fabry disease (13). However, no precise molecular mechanism for cardiac hypertrophy and renal manifestations has been proposed. The goal of our research was to identify the responsible signaling pathway and modulate it for therapeutic purposes.

In this study, we characterized the mouse model of Fabry disease (α-gal A knockout mouse) (14) and identified several previously unreported disease phenotypes. These phenotypes provided a clue to abnormal androgen receptor (AR) signaling and indicated the possibility of mitigating the disease manifestations in a mouse model of Fabry disease.

### Results

#### Phenotypes of Fabry mice

We found that both hemizygous male and homozygous female Fabry mice develop late-onset cardiac hypertrophy. Compared with wild-type (WT) controls with the same strain background, hemizygous male Fabry mice had significantly increased heart-to-body weight ratio at 12 and 18 months of age, but not at 5 months (Fig. 1A). Echocardiography showed significantly increased left ventricular (LV) wall thickness and LV mass in 18-month-old male Fabry mice (Fig. 1B and C). There was no change in LV diameters (diastolic, 3.1 ± 0.3 mm in WT, 3.1 ± 0.2 mm in Fabry) and percent fractional shortening (53.4 ± 3.7% in WT, 55.3 ± 5.7% in Fabry) (n = 7). Expression levels of markers of cardiac hypertrophy, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and α- and β-myosin heavy chain (MHC) in heart tissues were analyzed by quantitative RT–PCR. ANP was significantly increased in male Fabry mice in an age-dependent manner (Fig. 1D). There was no significant change in BNP and α- and β-MHC (Supplementary Material, Fig. S1A–D). It is known that activated Akt is associated with cardiac hypertrophy (15). Akt activation requires the phosphorylation of the serine residue at position 473 and of the threonine residue at position 308. We found significantly increased phosphorylated Akt (Ser473) in male Fabry mouse hearts compared with WT controls (Fig. 1E). Parallel to the findings in heart mass and ANP, the increased phosphorylation of Akt was seen at 18 months, but not at 5 months of age. Masson’s Trichrome staining revealed no interstitial fibrosis.

![Figure 1. Phenotypes of hemizygous male Fabry mice.](image-url)
in either Fabry mice or WT at any analyzed time point. Homozygous female Fabry mice had similar age-dependent hypertrophic findings (Supplementary Material, Fig. S2A–C).

We also found that both sexes of Fabry mice develop progressive renal hypertrophy. Male Fabry mice had remarkably increased kidney-to-body weight ratio after 12 months of age (Fig. 1F). Histological analysis showed increased width of the renal cortex and thickness of the proximal tubules in 18-month-old male Fabry mice (Fig. 1G and H). Fabry mice had a significantly increased number of intracellular vacuoles in proximal tubule epithelial cells (Fig. 1I). Urine albumin/creatinine ratio and plasma creatinine and BUN levels in male Fabry mice were similar to WT mice at 14–16 months. Homozygous female Fabry mice had enlarged kidneys with the same time course as male Fabry mice (Supplementary Material, Fig. S2D).

In addition, we found significantly increased testicular weight in Fabry mice (Fig. 1J). Fat deposition was significantly decreased in both male and female Fabry mice compared with WT controls at 12 months (Fig. 1K and Supplementary Material, Fig. S2E).

**Increased AR activity in Fabry mice and Fabry patients’ cells**

We found that all the above abnormalities in Fabry mice can be explained by excess androgen action. It is known that androgens cause cardiac hypertrophy (16,17), as well as increased kidney size (18,19). The histological basis of androgen-induced kidney hypertrophy is hypertrophied proximal tubules (19). Androgens may regulate testicular size, as suggested by the small testes in AR-deficient mice (20). Androgens inhibit fat deposition by blocking a signal transduction pathway that normally supports adipocyte function (21). AR-deficient mice develop obesity and kidney atrophy (22).

We therefore tested whether androgen action is enhanced in Fabry mice. Androgens exert their biological roles mainly through AR, which is a ligand-dependent transcription factor. The binding of androgen to the AR results in translocation of AR from the cytosol into the nucleus, where AR binds to androgen response element (ARE) and regulates the expression of androgen-regulated genes (or AR-target genes) (23). Transcription levels of androgen-regulated genes reflect the activity of AR signaling. Insulin-like growth factor 1 (IGF-1) is a well-characterized androgen-regulated gene, which is positively regulated by AR (24). The expression of IGF-1 was significantly upregulated in male Fabry mouse heart (Fig. 2A). Transforming growth factor-β1 (TGF-β1), which is negatively regulated by AR (25), was downregulated in Fabry mouse heart (Fig. 2A). β-Glucuronidase (Gb3), kidney androgen-regulated protein (Kap) and acyl-CoA synthetase medium-chain family member 3 (Acsm3, also known as SA gene), the most established androgen-regulated genes in mouse kidneys (19,26,27), were all significantly upregulated in male Fabry mouse kidneys (Fig. 2A). In addition to mRNA levels, Gusb enzyme activity in kidneys was significantly increased in Fabry mice (Fig. 2B). The Gusb activity correlated well with its mRNA levels, suggesting that the increased activity is caused by upregulated gene transcription (Fig. 2C). Androgens increase urinary excretion of Gusb (18). Gusb activity in urine was significantly increased in male Fabry mice compared with WT controls (Fig. 2D). Collectively, these data strongly suggested that the activity of AR signaling is increased in male Fabry mouse hearts and kidneys.

We also tested whether AR signaling is abnormally activated in homozygous female Fabry mice. Consistent with that of male mice, cardiac expression of IGF-1 was significantly upregulated in female Fabry mice compared with WT controls (Fig. 2E). However, the regulation of androgen-regulated genes in female Fabry mouse kidneys differed clearly from that of the male Fabry mice. Gusb and Acsm3 mRNA levels were unchanged in homozygous female Fabry mouse kidneys relative to WT controls, and Kap transcription was significantly downregulated in female Fabry mice (Fig. 2F). Parallel to the mRNA level, kidney Gusb activity was unchanged in female Fabry mice.

The increased activity of AR signaling can be caused either by increased levels of ligands or increased transcriptional function of AR. We found blood testosterone levels to be similar in male Fabry mice compared with WT controls at both 2 and 12 months of age (Supplementary Material, Fig. S3A and B). This suggested that enhanced AR signaling in Fabry mice may be caused by increased AR activity.

To assess transcriptional activity of AR, we performed an AR reporter gene assay in primary cultured renal proximal tubules isolated from Fabry mice. The proximal tubules isolated from 12-month-old male Fabry and WT mice were infected with adenovirus encoding luciferase gene controlled by an ARE-containing promoter (Ad/MLUC7) (27). Dihydrotestosterone (DHT) could not induce luciferase expression through endogenous AR in either Fabry or WT-derived tubule cells (Fig. 2G), suggesting that AR expression level under this experimental condition is not sufficient to transactivate the reporter expression. When exogenous AR was co-expressed by Ad5hAR (27), DHT induced luciferase expression, and this AR-transactivation was moderately, but significantly, enhanced in Fabry mouse tubule cells compared with WT (Fig. 2G).

To further determine whether the aberrant AR signaling is also present in human patients, we tested Fabry patient-derived microvascular endothelial cell line (IMFE1) (28). IMFE1 cells have decreased α-gal A activity and accumulated Gb3. To obtain iso- genic ‘normal’ control cells, a normal α-gal A (GLA) cDNA was retrovirally introduced into IMFE1 cells (designated as IMFE1(α-gal +)). α-Gal A deficiency was corrected and Gb3 accumulation was cleared in IMFE1(α-gal +) cells compared with mock-treated IMFE1 cells [IMFE1(mock)] (Fig. 2H and I). To compare gene profile in this pair of diseased and ‘cured’ cells, we performed a PCR array, which constitutes 84 endothelial cell biology-related genes including prostate-specific antigen (PSA or KLK3), the best known androgen-regulated gene in humans (29). There were only two genes whose expressions significantly differed (3-fold difference) in IMFE1(α-gal +) and IMFE1(mock) cells, and interestingly, one of these was PSA. PSA mRNA level in IMFE1(mock) was 3.9-fold than that in IMFE1(α-gal +) cells (Fig. 2I). A complete list of the examined genes and their expression levels is included in Supplementary Material, Table S1.

**Gb3 accumulation is associated with upregulated AR transcription**

We asked whether the increased AR activity in Fabry mouse tissues is caused by increased AR gene expression. AR protein level was not increased in Fabry mouse hearts (Fig. 3A) and kidneys (not shown), suggesting that the higher gene expression level was not causal. Interestingly, however, we found increased AR mRNA levels in male Fabry mouse hearts and kidneys at most time points (Fig. 3B and C). AR mRNA in 12-month-old female Fabry mouse hearts trended towards a higher level relative to WT mice (Fig. 3D). AR mRNA level correlated well with expression levels of androgen-regulated genes in mouse tissues (Supplementary Material, Fig. S4A–D), suggesting a potential link between upregulated AR and the increased activity of AR signaling. To more directly determine the role of GSL on AR
transcription, a Gb3-loading study was carried out in HL-1 cells, an immortalized mouse cardiac cell line that retains a number of characteristics of cardiomyocytes (30). HL-1 cells have normal α-gal A activity and have no Gb3 detected by immunostaining (Fig. 3E). It is known that exogenously added GSLs can be accumulated in plasma membrane and intracellular membrane systems in cultured cells with normal lysosomal enzymes (31). Gb3 loading led to robust cell-associated (presumably plasma membrane and endosomes) accumulation of Gb3 in HL-1 cells (Fig. 3E), and Gb3-loaded HL-1 cells had significantly increased AR mRNA expression compared with mock-treated cells (Fig. 3E). AR transcription was also upregulated in Fabry patients’ cells. AR mRNA level was slightly but significantly increased in male Fabry patients’ fibroblasts compared with healthy controls’ cells (Fig. 3F) and was >14 times higher in IMFE1(mock) cells than in IMFE1(α-gal+) (Fig. 3G).

Blockade of AR signaling ameliorates disease phenotypes in male Fabry mice

We next examined whether increased activity of AR signaling is responsible for disease pathogenesis. To this end, we tested whether blockage of AR signaling by castration can prevent the development of disease phenotype. Fabry male mice were castrated at 2 months of age (an asymptomatic stage), and the effects of castration were evaluated at 18 months of age.

Figure 2. Increased activity of AR signaling in Fabry disease. (A) mRNA levels of IGF-1 and TGF-β1 in hearts, and Gusb, Kap and Acsm3 in kidneys in 18-month-old hemizygous male Fabry mice and WT controls measured by real-time RT-PCR (n = 6–7). (B) Gusb activities in kidneys from 18-month-old male Fabry mice and WT controls (n = 7). (C) Correlation between Gusb mRNA levels and Gusb activities in 18-month-old male mouse kidneys. (D) Gusb activities in urine from 14- to 16-month-old male Fabry mice and WT controls (n = 10–14). (E and F) mRNA levels of cardiac IGF-1 (E) and kidney Kap (F) in homozygous female Fabry mice and WT controls (n = 6–8). (G) AR reporter gene assay in suspension cultures of renal proximal tubules using adenovirus containing MMTV-Luciferase with and without co-expression of exogenous AR by Ad5shAR (n = 4). (H) Corrected enzyme deficiency in IMFE1(α-gal+) cells. α-gal A protein detected by western blot (upper) and enzyme activities (lower, unit: nmol/mg/h) are shown. (I) Gb3 immunostaining (red, Gb3; blue, DAPI nuclear counterstain). Gb3 accumulation was cleared in IMFE1(α-gal+) cells.

(J) PSA mRNA levels in IMFE1(mock) and IMFE1(α-gal+) cells (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 determined by t-test.
As expected, blood testosterone levels in castrated male Fabry mice were markedly decreased and were even lower than in intact female Fabry mice (Fig. 4A). Compared with untreated male Fabry mice, the mRNA levels of IGF-1 decreased and TGF-β1 increased in castrated Fabry mouse hearts (Fig. 4B). Expression of Gusb, Kap and Acsm3 all decreased in castrated Fabry mouse kidneys (Fig. 4C). Gusb activity in kidney decreased to the untreated WT level as well (Fig. 4D). Abolished expression of Kap and Acsm3 in castrated mice indicated that AR signaling was blocked by castration. Moreover, the restored expression of IGF-1, TGF-β1 and Gusb by castration suggested that the aberrant expression of these genes in Fabry mouse hearts and kidneys (Fig. 2A) is indeed the consequence of enhanced AR signaling. Castration completely prevented the development of cardiac hypertrophy in Fabry mice. Heart-to-body weight ratio, LV wall thickness measured by echocardiography and cardiac ANP mRNA levels in castrated Fabry mice were at the levels of untreated WT mice (Fig. 4E–H). Akt phosphorylation in castrated Fabry mouse hearts decreased significantly compared with untreated Fabry mice (Fig. 4I). Kidney weight of castrated Fabry mice remained at WT levels as well (Fig. 4J). More importantly, in WT mice, all these parameters were unchanged by castration (Fig. 4E–J), suggesting that the effect of androgen depletion in Fabry mice is disease process specific, rather than a universal effect on normal heart and kidney development.

It is known that synthesis of Gb3 in murine kidneys is upregulated by androgens (32). To address whether the effect of castration on disease phenotypes of Fabry mice is through reduced synthesis of Gb3, we measured Gb3 in mouse tissues. Gb3 was significantly reduced in castrated WT kidneys as expected (Fig. 4L) but was unchanged in the hearts and kidneys of castrated Fabry mice (Fig. 4K and L), indicating that the abolished cardiac and kidney hypertrophy in castrated Fabry mice was not due to reduced Gb3 in these organs.

Next, we tested whether castration can correct preexisting cardiac and renal pathology in Fabry disease. Fabry mice were castrated at 12 months of age when cardiac and kidney hypertrophy is apparent, and the effects were evaluated at 18 months of age. Heart weight, ANP mRNA level and kidney weight in castrated Fabry mice were reduced to the levels of untreated WT (Supplementary Material, Fig. S5A–C). Histologically, renal cortex width and proximal tubule wall thickness in castrated Fabry mice were reduced to untreated WT levels (Supplementary Material, Fig. S5D and E).

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**Figure 3.** Upregulated AR mRNA expression in Fabry disease. (A) AR in mouse heart homogenates (18-month-old males) was enriched by immunoprecipitation and was detected by western blot. Whole lysate of mouse testis (25 μg total protein) was used as positive control. Data represent two independent experiments. (B and C) AR mRNA levels in male mouse heart (B) and kidney (C) tissues measured by real-time RT–PCR (n = 6–9). (D) AR mRNA levels in 12-month-old female mouse hearts (n = 6). (E) The role of Gb3 on AR mRNA expression in HL-1 cells. Left: immunostaining against Gb3; the most Gb3-loaded HL-1 cells showed cell-associated positive signal (red, Gb3; blue, DAPI nuclear counterstain). Right: AR mRNA levels in HL-1 cells cultured in the presence or absence of 20 μM of Gb3/albumin complex for 5 or 21 days (n = 3–6; statistical analysis, Gb3-loaded versus mock-treated). (F) AR mRNA levels in skin fibroblasts obtained from male adult Fabry patients and healthy controls (n = 27 and 11, respectively). (G) AR mRNA levels in IMFE1(mock) and IMFE1(α-gal+) cells (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 determined by t-test.
supplementation of exogenous androgen (DHT) in castrated Fabry mice restored all these abnormalities (Supplementary Material, Fig. S5A–E). This further confirmed the pathogenic role of AR signaling in Fabry disease. The vacuolization of proximal tubules, which may reflect glycolipid storage, was not changed by castration.
We further tested whether pharmacological blockade of AR signaling can ameliorate disease phenotypes. Eighteen-month-old Fabry mice were treated with flutamide for 7 weeks by daily subcutaneous injection at a dose of 25 mg/kg/day. Flutamide is a potent non-steroidal AR-antagonist; it competes with testosterone and DHT for binding to AR. Gusb activity in kidney decreased in both flutamide-injected WT and Fabry mice, with more significant decrement in Fabry mice (Fig. 5A) suggesting attenuated activity of AR signaling. Flutamide treatment resulted in decreased heart-to-body weight ratio and LV mass as well as decreased kidney-to-body weight ratio in Fabry mice compared with placebo-treated Fabry mice (Fig. 5B–D). Consistent with the findings in the castration study (Fig. 4), these parameters in WT mice were not changed by flutamide treatment (Fig. 5B and D).

Taken together, these results suggested that AR signaling contributes to cardiac and renal hypertrophy in male Fabry mice and that blockade of AR signaling is able to prevent and correct these disease phenotypes.

Discussion

As is the case in most lysosomal storage disorders, the downstream pathogenic mechanism of Fabry disease is complex. Impaired membrane trafficking, autophagy, signaling transduction and inflammation process, and cellular injury due to toxic metabolites (such as lyso-sphingolipids) are some of the common cellular abnormalities of sphingolipidoses (31,33–35). In the present study, we proposed a new mechanism, i.e. that enhanced AR signaling contributes to some of the clinical manifestations of Fabry disease.

The role of androgen action in cardiac hypertrophy is well established. Androgens induce a hypertrophic response in cultured cardiomyocytes in an AR-dependent manner (16). Testosterone administration in rats causes cardiac hypertrophy associated with increased IGF-1 mRNA and Akt phosphorylation in the heart with no significant change in ANP and β-MHC expression and cardiac fibrosis (17). Conversely, AR-deficient mice show smaller heart size and reduced hypertrophic response to angiotensin II stimulation (36). Angiotensin II causes more prominent cardiac fibrosis and systolic dysfunction in AR-deficient mice than in WT mice with increased TGF-β1 mRNA and Smad2 activation (36). These studies suggest that the androgen/AR system induces or promotes hypertrophic response probably through the IGF-1/Akt signaling pathway but, on the other hand, suppresses the development of fibrosis and systolic dysfunction via decreased activity of TGF-β1/Smad signaling. The pattern of androgen/AR-induced cardiac hypertrophy (i.e. less significant fetal gene upregulation, cardiac impairment and fibrosis) clearly differs from that seen in experimental models of pressure overload-induced cardiac hypertrophy (37). Cardiac phenotypes of Fabry mice are consistent with the pattern of androgen-induced hypertrophy. The generally preserved ejection fraction in Fabry patients (4) is also in agreement with this pattern. A difference between mice and humans is cardiac fibrosis, which is often seen in Fabry patients. Possible reasons for this discrepancy are the longer disease course in patients (years or decades) and/or...
differences in signaling systems that are modulated by AR in the mouse and human heart.

It has long been recognized that the kidney is an androgen-sensitive organ (18,19). Androgens cause hypertrophy and increased expression of Gusb and other specific proteins in murine kidneys (18,19,26). Proximal tubule cells are the predominant cell type responsible for androgen action in kidneys with respect to renal hypertrophy and induction of androgen-regulated genes (19). Our results revealed that the cellular basis of kidney enlargement in Fabry mice is hypertrophied proximal tubule cells mediated by AR signaling. Enlarged kidneys in Fabry patients (5) are probably caused by the same mechanism. Activated proximal tubules can be involved in kidney disease pathogenesis via various mechanisms including generation of reactive oxygen species and inflammatory cytokines (38). However, because Fabry mice do not develop renal dysfunction, it is difficult to investigate whether and how the proximal tubule hypertrophy contributes to Fabry kidney disease. A recent study reported that Fabry mice with increased Gb3 synthesis develop renal impairment associated with significantly increased kidney-to-body weight ratio in both sexes (39). It will be important to study the effects of AR-targeting treatment in renal dysfunction in this Fabry mouse strain.

Although our interventional studies were focused on male Fabry mice, we hypothesize that AR contributes to Fabry disease in females as well. First, although androgens are not dominant sex hormones in females, AR activity in hearts and kidneys of female mice is similar to that in males (40). Thus, aberrant AR signaling can be pathogenic in females. Second, homozygous female Fabry mice develop similar phenotypes to those found in male Fabry mice, suggesting that both sexes share the same pathogenic mechanism. Third, the upregulated IGF-1 suggested increased AR activity in female Fabry mouse hearts. However, given the different hormone systems, it is possible that some different mechanisms may be operational in females compared with males. This was suggested by the sexual dimorphism in the gene expression profile of Fabry mouse kidneys, typically in Kap expression. Kap is strictly regulated in a sex-specific manner (41). In males, it is expressed in the S1/S2 segments of proximal tubules and is regulated by androgens. In contrast, in females it is exclusively expressed in the S3 segment and is regulated not only by androgens, but also by thyroid hormone and estrogen. Thus, expression of Kap in female kidneys is the result of combined effects of multiple hormones and may not be a good indicator of AR activity.

It should be noted that the conclusion obtained from homozygous female Fabry mice cannot be directly applied to the pathogenesis of cardiac hypertrophy in heterozygous female patients, in which the heart is a mosaic of normal and affected cells resulting from random X-chromosome inactivation. It is intriguing as to how dysfunction of the entire organ occurs when only some of the cells are affected. One potential mechanism can be paracrine action of IGF-1, i.e. the increased secretion of IGF-1 (and/or other growth factors) from affected cardiomyocytes may act on adjacent normal cardiomyocytes, causing their hypertrophic growth. Further investigations should be conducted in heterozygous female Fabry mice to address this possibility.

Upregulated PSA in Fabry patients’ endothelial cells suggested that increased AR activity may be also present in human patients. However, we cannot rule out the possibility that there may be some species-specific disease mechanisms that are related to a different glycolipid metabolism in mice and humans, e.g. the less profound GSL storage in Fabry mouse endothelial and cardiac cells than in patients (42). Further investigations will be necessary to gain more direct evidence for enhanced AR signaling in Fabry patients.

The mechanism through which Gb3 accumulation causes increased activity of AR remains unclear. Transcriptional activity of AR is subjected to complex regulation. It is regulated by a number of coactivators and corepressors that influence functional properties of AR, including ligand selectivity and DNA binding capacity (23). The increased transactivation activity of exogenous AR in the reporter gene assay suggests abnormal AR coactivators in Fabry mouse cells. AR activity is modulated by phosphorylation of AR or its coactivators. Several signal transduction pathways such as MAPK, Akt and PKC are known to be involved in this phosphorylation (43,44). On the other hand, our data showed increased AR mRNA in Fabry disease. AR promoter activity is regulated by several transcription factors (45–48), whose activities are modulated by different signaling pathways such as Akt and Wnt. We hypothesize that both increased AR mRNA and activated AR signaling might be mediated by the same upstream signaling pathway. Close correlation between mRNA levels of AR and androgen-regulated genes (Supplementary Material, Fig. S4) supports this possibility. If so, analysis of AR promoter activity in cultured cells might facilitate identification of the signaling pathway responsible for the AR activation.

The blocking of AR signaling was able to ameliorate disease phenotypes in male Fabry mice despite the persistence of α-gal A deficiency and GSL storage suggesting that AR signaling is an important downstream pathway to GSL storage and can be a useful molecular target for therapy. The reversal of cardiac and renal hypertrophy in castrated or flutamide-treated Fabry mice suggested that AR signaling plays roles not only in development but also maintenance of these disease phenotypes. Although we used flutamide in animal studies, because of the agonist properties of flutamide (49), the second-generation antiandrogens such as MDV3100 (50) would be more suitable for potential therapy in humans. AR pathway-targeted approaches have been extensively studied for treating prostate cancer (51); thus, there are a number of agents available for clinical use. These strategies may be used as new therapy for Fabry disease or can be used in combination with preexisting therapies (e.g. ERT) to obtain better therapeutic efficacy.

Materials and Methods

Mice and treatments

All mice used in this study were maintained in our laboratory under standard housing conditions. Fabry mice (14) were produced by breeding pairs of hemizygous males and homozygous females. To determine genetic background, we performed microsatellite analysis (by Charles River), and the performed showed that Fabry mice are mixtures of C57BL/6J and 129 strains with ~75% of C57BL/6J strain background. WT mice with the same strain background were used as controls throughout the studies. All procedures performed on the mice were reviewed and approved by the Institutional Animal Care and Use Committee of Baylor Research Institute. Castration was performed under anesthesia using ketamine and xylazine. For androgen replacement, time-release DHT pellets (25 mg/pellet for 90 days release, Innovative Research of America) were implanted subcutaneously 1 week after castration. The pellets were replaced every 3 months. For flutamide treatment, flutamide dissolved in 10% ethanol in corn oil was injected subcutaneously daily at a dose of 25 mg/kg/day. Control mice received vehicle injection.

Echocardiography

In vivo cardiac morphology was assessed by transthoracic echocardiography (Vevo 2100, VisualSonics) in conscious mice.
Systolic and diastolic LV internal dimensions and LV wall thickness were averaged from 3–6 beats. Thicknesses of anterior and posterior walls were averaged. The analysis was performed in a manner blind to genotype and treatment conditions.

**Kidney histology**

Kidneys were cut transversely, fixed in 10% formaldehyde and paraffin embedded. Five-micrometer-thick sections were made and stained with PAS. Photomicrographs were analyzed using imaging software to assess cortical width and proximal tubule wall thickness. The analysis was performed by a veterinarian pathologist (M.W.C.) in a blinded manner.

**Urine analysis**

Mouse overnight urine samples were collected using metabolic cages and were stored at −80°C until use. Urine albumin concentration was measured using a mouse albumin ELISA kit (Bethyl Lab). For Gusb activity, 10 μl of urine was directly used for enzyme assay. Urinary albumin concentrations and Gusb enzyme activities were normalized by creatinine concentrations that were measured using COBAS Integra 400.

**Cell culture and treatments**

Skin fibroblasts obtained from male Fabry patients (n = 27, mean donor age 39.7 with average residual α-gal A activity in leukocytes 3.6% of normal) and male normal controls (n = 11, mean age 27.3) were cultured in 10% fetal bovine serum (FBS) in DMEM.

Mouse cardiac muscle cell line, HL-1 (30), was cultured in Claycomb basal medium (Sigma) supplemented with 10% FBS, 0.1 mM norepinephrine and 2 mM L-glutamine. Gb3 loading was performed as previously described (28). The cells were incubated with 20 μM Gb3/albumin complex for 5 or 21 days before analysis. Gb3 accumulation in the cells was confirmed by Gb3 immunostaining. For mock treatment, solvent (DMSO) was used in place of Gb3.

IMFE1 cells, a Fabry patient-derived microvascular endothelial cell line (28), were cultured in EGM2MV medium (Lonza). To correct the enzyme deficiency, IMFE1 cells were infected with a MPP retroviral vector encoding normal α-gal A with co-expression of GFP through an internal ribosome entry site (IRES) (52). Control cells [IMFE1(mock)] were infected with an empty retroviral vector (the same backbone without α-gal A gene). The infected cells were purified by selecting GFP-positive cells using fluorescence-activated cell sorting. After sorting, the GFP-positive cell purity was ~93% for both IMFE1(α-gal+) and IMFE1(mock).

**Culture of proximal tubules and AR reporter gene assay**

Renal proximal tubules were isolated from 12-month-old male Fabry and WT mice as described previously (53) with modifications. Briefly, renal cortices were dissected and minced into small pieces (~1 mm³) and were digested with 1 mg/ml type-2 collagenase (Invitrogen) at 37°C for 30 min in the presence of 1 mg/ml soybean trypsin inhibitor (Sigma). The same volume of horse serum was added, and the suspension was mixed by vortex for 30 s. The suspension was allowed to sediment for 1 min, and the proximal tubule-enriched supernatant was centrifuged at 200g for 7 min. After being washed with HBSS to remove serum, the tubule fragments were suspended in DMEM/F12 (1:1) supplemented with 5% charcoal-stripped FBS (cs-FBS) and were plated on 48-well plates. Recombinant adenoviruses Ad/MLUC7 (reporter vector containing MMTV-Luciferase (27); 1 × 10⁶ pfu) and Ad-LacZ (internal control for gene transduction efficiency; 0.25 × 10⁶ pfu) were added to each well. AdShAR (27) was also added to some wells (0.25 × 10⁶ pfu/well) to express endogenous AR. The infection was performed in the volume of 0.1 ml/well for 3 h with occasional agitation. After that, 5% cs-FBS in DMEM/F12 and DHT were added to achieve the final volume of 0.2 ml/well with or without 10 nm DHT. Two days later, the tubules were harvested and lysed for reporter assays. Luciferase activity was determined using luciferase reporter assay kit (Promega) and a luminometer (GloMax 20/20, Promega). β-galactosidase activity was measured using the colorimetric method with 1 mg/ml o-nitrophenyl-β-D-galactopyranoside at pH 7.0. Luciferase activity was normalized by β-galactosidase activity.

**Quantitative RT–PCR**

Quantitative RT–PCR was performed as described previously (28), using pre-designed TaqMan probe/primer sets (Applied Biosystems) or SYBR Green primers (Sigma). 18S rRNA was used as internal control.

**Western blot and immunoprecipitation**

Western blot analysis was performed as described previously (28). Primary antibodies used were rabbit antibodies to phospho-Akt (Ser473), total Akt (Cell Signaling), AR (N-20, Santa Cruz) and human α-gal A (Shire), and goat antibody to GPDH (Santa Cruz). Intensity of the bands was analyzed using ImageJ software. Because of the low abundance, AR protein in mouse heart and kidney lysates could not be directly detected by western blot, and thus, AR was enriched by immunoprecipitation. In brief, mouse tissues were homogenized in RIPA buffer with protease inhibitors (Santa Cruz). Lysates containing 1 mg total protein were incubated with 1 μg rabbit antibody to AR (N-20, Santa Cruz) overnight at 4°C followed by mixing with 20 μl Protein A/G PLUS-Agarose beads (Santa Cruz) for 1 h at 4°C. For negative control, normal rabbit IgG was used. The beads were washed four times with 0.5% Triton-X in PBS, and the proteins on the beads were analyzed by western blot using the antibody to AR.

**Testosterone level**

Plasma level of total or free testosterone was measured using radioimmunoassay kit (Siemens Diagnostics).

**Analysis of Gb3 levels**

Gb3 levels in mouse tissues were measured by mass spectrometry as described previously (54). Gb3 levels in cultured cells were analyzed by immunostaining using monoclonal antibody to Gb3 as described (28).
Lysosomal enzyme assays
Activities of α-gal A and GUSB were determined by the fluorimetric method using 4-methylumbelliferyl-labeled α-D-galactopyranoside and β-D-glucuronide, respectively.

Statistical analysis
Data were presented as mean ± s.e.m. Statistical significance was determined by the Student’s t-test. One-way ANOVA followed by Bonferroni’s test was used for multiple comparisons.

Supplementary Material
Supplementary material is available at HMG online.

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References


