Nephrol Dial Transplant (2011) 26: 1797–1802
doi: 10.1093/ndt/gfq306
Advance Access publication 26 May 2010

Globotriaosylsphingosine actions on human glomerular podocytes: implications for Fabry nephropathy

Maria D. Sanchez-Niño[1], Ana B. Sanz[2], Susana Carrasco[1], Moin A. Saleem[3], Peter W. Mathieson[3], José M. Valdivielso[4], Marta Ruiz-Ortega[1], Jesus Egido[1] and Alberto Ortiz[1]

[1]Nefrología, Fundación Jiménez Díaz, Universidad Autonoma de Madrid and Instituto Reina Sofia de Investigaciones Nefrológicas-IRSIN, Madrid, Spain, [2]Servicio de Nefrologia, Fundacion para la Investigacion Biomedica del Hospital Universitario La Paz, Madrid, Spain, [3]Academic and Children's Renal Unit, University of Bristol, Bristol, UK and [4]Experimental Nephrology Laboratory, Institut de Recerca Biomedica de Lleida (IRBLLEIDA), Spain

Correspondence and offprint requests to: Alberto Ortiz; E-mail: aortiz@fjd.es, albertortiza@terra.es

Abstract

Background. Transforming growth factor-β1 (TGF-β1) and the macrophage inhibitory factor receptor CD74 link the metabolic disorder with tissue injury in diabetic nephropathy. Fabry disease is an X-linked lysosomal glycosphingolipid storage disorder resulting from a deficient activity of α-galactosidase A that leads to proteinuric renal injury. However, the link between the metabolic abnormality and renal injury is poorly characterized. Globotriaosylsphingosine (lyso-Gb3) was recently identified as a bioactive molecule accumulating in Fabry disease. We hypothesized that lyso-Gb3 could modulate the release of secondary mediators of injury in glomerular podocytes and that recently described nephroprotective actions of vitamin D receptor activation in diabetic nephropathy may apply to lyso-Gb3.

Methods. Real time RT-PCR, ELISA and Western blot were used to study the biological activity of lyso-Gb3 in cultured human podocytes and potential modulation by vitamin D receptor activation.

Results. In human podocytes, lyso-Gb3 dose and time dependently increased the expression of TGF-β1, extracellular matrix proteins (fibronectin and type IV collagen) and CD74. TGF-β1 mediated lyso-Gb3 effects on extracellular matrix production. Vitamin D receptor activation with paricalcitol or calcitriol prevented the increase in TGF-β1, CD74 and extracellular matrix induced by lyso-Gb3.

Conclusions. Lyso-Gb3 may have a role in glomerular injury in Fabry disease by promoting the release of secondary mediators of glomerular injury common to diabetic nephropathy. These effects are prevented by paricalcitol, raising the issue of vitamin D receptor activation as potential adjunctive therapy in Fabry nephropathy.

Keywords: fabry disease; glomerular injury; podocytes

Introduction

Fabry disease is an X-linked storage disorder resulting from a deficient activity of α-galactosidase A [1,2]. As a consequence of the enzymatic deficiency, there is a progressive accumulation of glycosphingolipids, predominantly of the enzyme substrate globotriaosylceramide (Gb3), in lysosomes, other cellular compartments and the extracellular space [3]. Initially, reversible changes, such as glycosphingolipid accumulation, are followed by irreversible tissue injury and, ultimately, by organ failure which may be life-threatening when affecting the kidney, heart or central nervous system [4,5]. However, the pathogenic link between the metabolic abnormality (glycosphingolipid accumulation) and tissue injury is unclear.

In Fabry nephropathy, glycolipids deposit in glomerular (particularly podocytes), tubular and vascular cells leading to glomerulosclerosis, tubular atrophy, interstitial fibrosis and inflammation [6,7]. Fabry nephropathy resembles diabetic nephropathy (DN) in the metabolic origin and in the clinical course characterized by increasing proteinuria and progression of renal dysfunction at a similar rate of loss of glomerular filtration rate of −10 to −12 mL/min/year [8–11]. Early enzyme replacement therapy (ERT) stabilizes renal function, but ERT does not influence proteinuria, and may not prevent progressive renal deterioration in patients with glomerulosclerosis or proteinuria >1 g/day [4,12]. As it is the case for DN, Fabry nephropathy appears to have a point of no return, beyond which the metabolic abnormality may no longer be the main driver of progression [4,13]. At this stage, secondary mediators of renal injury take centre stage, and a better knowledge of these mediators may provide additional therapeutic tools to slow progression of more advanced cases of renal injury. In this regard, and based on their effectiveness in DN, adjunctive antiproteinuric therapy with angiotensin-converting enzyme

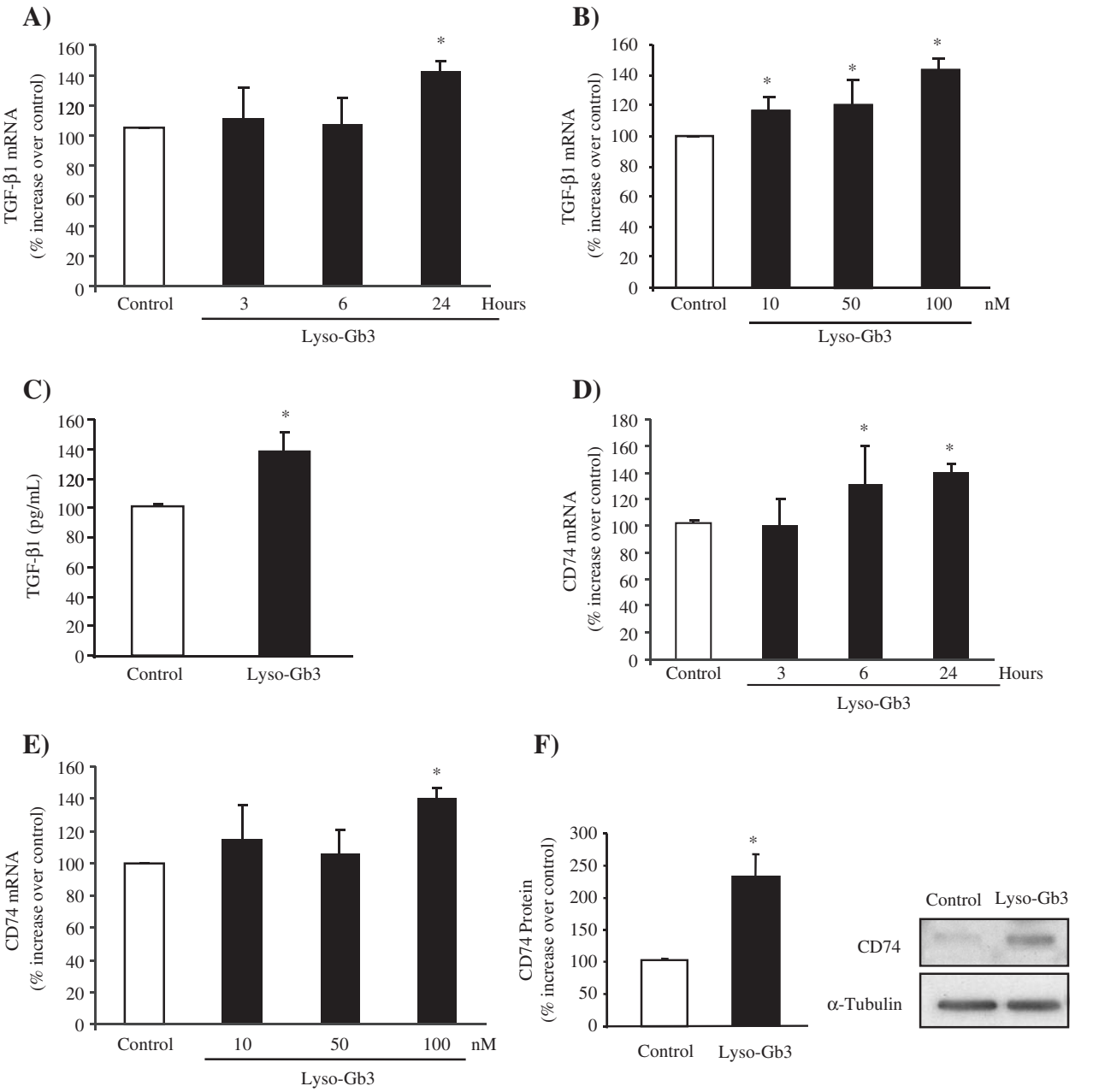

Fig. 1. Lyso-Gb3 increases TGF-β1 and CD74 expression in human podocytes. (**A**) Time-dependent increase of TGF-β1 mRNA in cultured human podocytes after 100 nM lyso-Gb3 incubation and (**B**) dose–response increase of TGF-β1 mRNA after a 24-h exposure to lyso-Gb3. Real-time RT–PCR. Mean±SD of three independent experiments. *P<0.002 vs. control. (**C**) Supernatant TGF-β1 protein ELISA at 24 h. *P<0.01 vs. control. (**D**) Time-dependent increase in CD74 mRNA expression in human podocytes treated with 100 nM lyso-Gb3 for indicated time periods. Real-time RT–PCR. Mean±SD of four independent experiments. *P<0.01 vs. control. (**E**) Dose–response increase in CD74 mRNA at 24 h after stimulation with lyso-Gb3 at different concentrations. Real-time RT–PCR. Mean±SD of three independent experiments. *P<0.01 vs. control. (**F**) Increase in CD74 protein expression in cells treated with 100 nM lyso-Gb3 for 24 h. Representative western blot and densitometric quantification. Mean±SD of three independent experiments. *P<0.005 vs. control.

inhibitors (ACEI) or angiotensin receptor blockers (ARBs) has been advocated, and a small, open-label trial provided encouraging results [4,14]. Paricalcitol, a selective vitamin D receptor (VDR) activator, has been reported to reduce proteinuria in DN but has not been tested in Fabry nephropathy [15,16]. However, the secondary mediators leading to tissue injury in Fabry disease are poorly understood. Similar to diabetes, the initial metabolic derangement may promote the production of secondary mediators of injury that lead to fibrosis, parenchymal cell loss and inflammation. Although Gb3 accumulation is widespread, serum Gb3 or Gb3 deposits do not necessarily correlate with clinical manifestation [17]. Recently, high serum concentrations of a biologically active lipid metabolite, globotriaosylsphingosine (lyso-Gb3), have been observed in Fabry patients [17]. Lyso-Gb3 promoted vascular smooth muscle cell proliferation, suggesting a role in the pathogenesis of Fabry disease. However, it is unknown whether lyso-Gb3 has

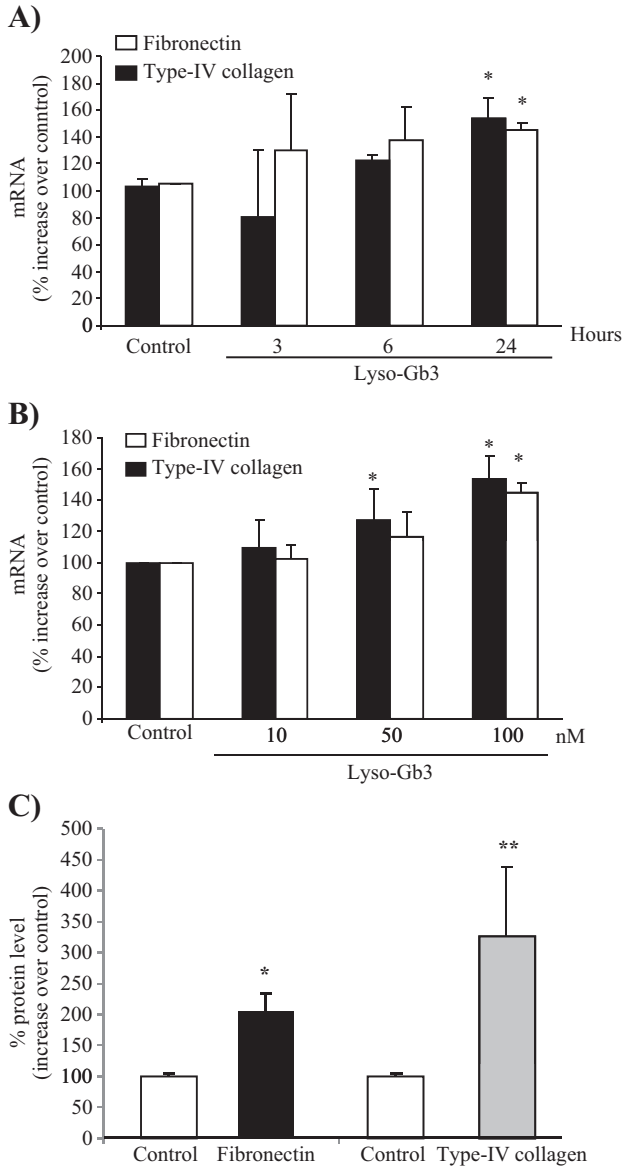

Fig. 2. Lyso-Gb3 induces extracellular matrix protein expression in cultured human podocytes. (**A**) Time-course of fibronectin and type IV collagen mRNA expression in human podocytes treated with 100 nM lyso-Gb3. Mean±SD of four independent experiments. *P<0.004 vs. control. (**B**) Dose–response after a 24-h stimulation with lyso-Gb3. Real-time RT–PCR. Mean±SD of four independent experiments. *P< 0.009 vs. control. (**C**) Cells were stimulated with 100 nM lyso-Gb3 for 24 h. Densitometric quantification of western blot. Mean±SD of three independent experiments. *P<0.01 vs. control, **P<0.04 vs. control.

biological actions on podocytes, which are the key cells in proteinuric kidney diseases, including DN [18].

We hypothesized that lyso-Gb3 may have biological actions on cultured podocytes, leading to the release of secondary mediators that have been previously implicated in the pathogenesis of other metabolic nephropathies, such as DN. Thus, we have explored the modulation by lyso-Gb3 of classical mediators of DN, such as transforming growth factor-β1 (TGF-β1) [11,19] and of novel mediators identified by unbiased genomic profiling, such as CD74 [20], and have explored novel potential therapeutic approaches, such as VDR activation.

Materials and methods

Cell culture and reagents

Human podocytes are an immortalized cell line transfected with a temperature-sensitive SV40 gene construct and a gene encoding the catalytic domain of human telomerase [20,21]. At a permissive temperature of 33°C, the cells remain in an undifferentiated proliferative state and divide. Raising the temperature to 37°C results in growth arrest and differentiation to the parental podocyte phenotype. Undifferentiated podocyte cultures were maintained at 33°C in RPMI 1640 medium with penicillin, streptomycin, insulin–transferrin–selenite (ITS) and 10% FCS. Once cells reached 70–80% confluence, they were fully differentiated by culture at 37°C for at least 14 days [20].

Cells were cultured in serum-free media 24 h prior to the addition of the stimuli and throughout the experiment. Inhibitors were added 1 h before lyso-Gb3 (Sigma, St. Louis, MO, USA): paricalcitol (10^{-7}M, Abbot), calcitriol (10^{-7}M, Sigma), neutralizing anti-TGF-β1 (10 μg/mL, R&D systems) or TGF-β1 superfamily type I activin receptor-like kinase receptor (ALK-5) inhibitor (TRI, SB431542, 10^{-6}M, Calbiochem) or vehicle. Inhibitors were not toxic (cell viability assay MTS-PMS, Promega or morphological assessment of apoptosis) and, in preliminary experiments, were shown to be active at the doses used [22,23].

Protein studies

For western blot, cells were homogenized in lysis buffer and separated by 10% or 12% SDS–PAGE under reducing conditions [20]. Primary antibodies were goat polyclonal anti-CD74 (1:500), rabbit polyclonal anti-collagen IV (1:500) (Santa Cruz) and mouse monoclonal anti-fibronectin (1:1000, Chemicom). Secondary antibodies were appropriate horseradish peroxidase-conjugated antibodies (1:2000, Amersham, Aylesbury, UK). Blots were then probed with mouse monoclonal anti-α-tubulin (1:2000, Sigma), and levels of expression were corrected for minor differences in loading.

TGF-β1 in cell culture supernatant was measured by ELISA (BD Biosciences, San Jose, CA, USA) [22].

Real-time reverse transcription–polymerase chain reaction

RNA was isolated by Trizol (Invitrogen, Paisley, UK) [20]. One microgram of RNA was reverse-transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol using the delta delta Ct method [20]. Expression levels are given as ratios to GAPDH. Pre-developed primer and probe assays (PDAR) were obtained for human GAPDH, VDR, CD74, collagen IV, fibronectin and TGF-β1 from Applied Biosystems.

Statistical analysis

Data are mean ± standard deviation. Mann–Whitney, two-sided *t*-test or one-way ANOVA was applied to detect differences between groups. A P-value <0.05 was considered statistically significant.

Results

Lyso-Gb3 increases podocyte TGF-β1, CD74 and extracellular matrix (ECM) protein expression

TGF-β1 is a key mediator of glomerulosclerosis and interstitial fibrosis released in response to high glucose [24]. CD74 is a candidate receptor mediator of podocyte injury in DN that is expressed in response to the metabolic derangement (high-glucose milieu) [20]. In podocytes, lyso-Gb3 increased TGF-β1 and CD74 mRNA levels in a time- (Figure 1A and D) and dose-dependent manner (Figure 1B and E). TGF-β1 protein was increased in the supernatants and CD74 protein in whole-cell extracts from stimulated cells (Figure 1C and F). Lyso-Gb3 increased the mRNA (Figure 2A and B) and protein (Figures 2C,

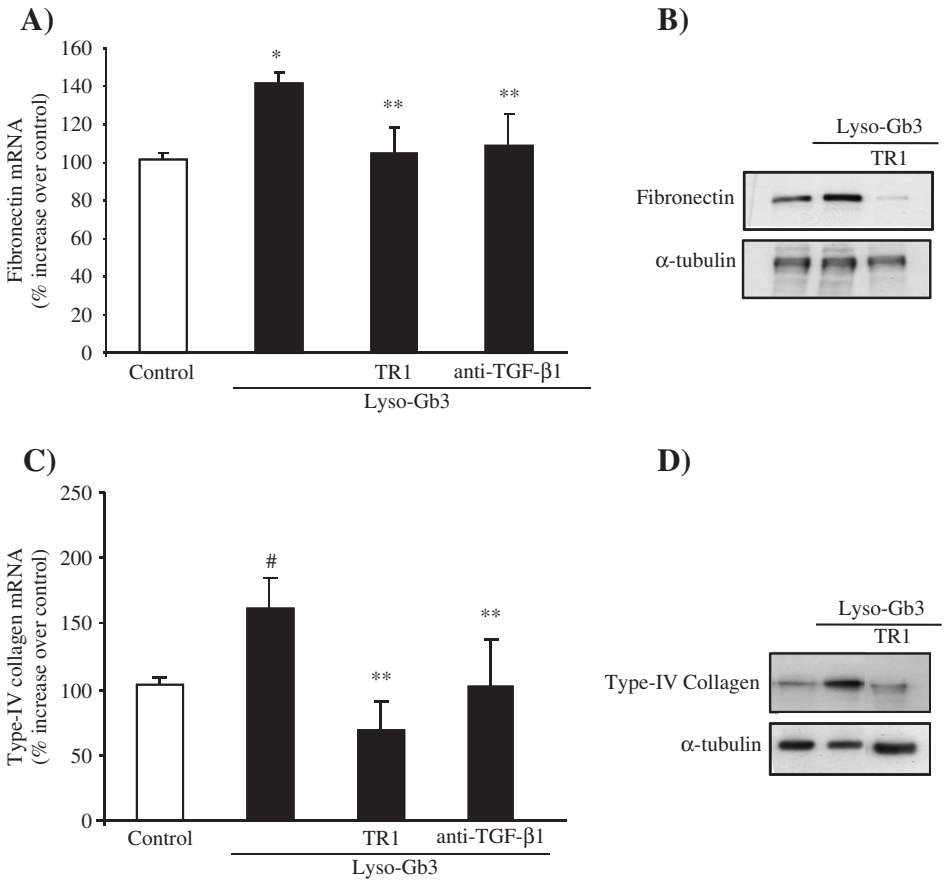

Fig. 3. TGF-β1 mediates the increased ECM component expression induced by lyso-Gb3. TGF-β1 was blocked by pre-treatment with 10 μg/mL anti-TGF-β1-neutralizing antibody or 10^{-5} mol/L TGF-β1 receptor inhibitor (TRI) for 1 h before 100 nM lyso-Gb3 stimulation for 24 h. Fibronectin (**A**, **B**) and type IV collagen (**C**, **D**) mRNA expression decrease after anti-TGF-β1 blockade. Mean±SD of four experiments. *P<0.002 vs. control; **P<0.01 vs. lyso-Gb3 alone, #P<0.03 vs. control. (**B**, **D**) Representative western blot.

and 3B and D) expression of ECM proteins like fibronectin and type IV collagen. The increase in ECM-coding mRNA levels was time- (Figure 2A) and dose-dependent (Figure 2B).

TGF-β1 mediates lyso-Gb3 regulation of ECM components

TGF-β1 promotes synthesis of ECM in renal cells, including glomerular podocytes [22,25]. Blockade of TGF-β1 by two different strategies (neutralizing anti-active TGF-β1 antibodies or TGF-β receptor 1 kinase inhibitor) [26] decreased fibronectin (Figure 3A and B) and type IV collagen (Figure 3C and D) mRNA and protein expression after a 24-h incubation with lyso-Gb3. These results suggest that fibrosis component induction by lyso-Gb3 is dependent on recruitment of endogenous TGF-β1.

VDR activation attenuates gene expression induced by lyso-Gb3

Paricalcitol is a VDR activator that may reduce proteinuria in patients with chronic kidney disease [15]. Human podocytes express VDR [27]. VDR mRNA expression was confirmed by qRT–PCR in our cultured podocytes (Figure 4A). Paricalcitol prevented the upregulation of CD74, TGF-β1, fibronectin and type IV collagen mRNA and protein expression induced by lyso-Gb3 in cultured podocytes (Figure 4B–D). Calcitriol, the natural VDR activator, also prevented lyso-Gb3 actions on podocytes (Figure 4C and D). VDR activators prevented the increase in TGF-β1 protein in supernatants from lyso-Gb3-stimulated cells (Figure 4D).

Discussion

The link between the metabolic abnormality in Fabry disease and kidney tissue injury is unclear. We now report that, in cultured podocytes, lyso-Gb3, at concentrations found in serum of Fabry patients, induces the production of mediators of glomerular injury shared by another metabolic nephropathy, DN, and that VDR activation prevents these effects. This information may be useful for the design of adjuvant therapies that improve outcomes in Fabry patients with established kidney disease.

Fabry disease is a rare disease [4]. This complicates the study of pathogenic factors and therapeutic options. Early ERT is safe and effective in preventing progression of kidney injury [4,12]. The lesser efficacy of ERT once Fabry nephropathy has caused proteinuria or glomerulosclerosis [12] raises the need for adjuvant therapies that cooperate with

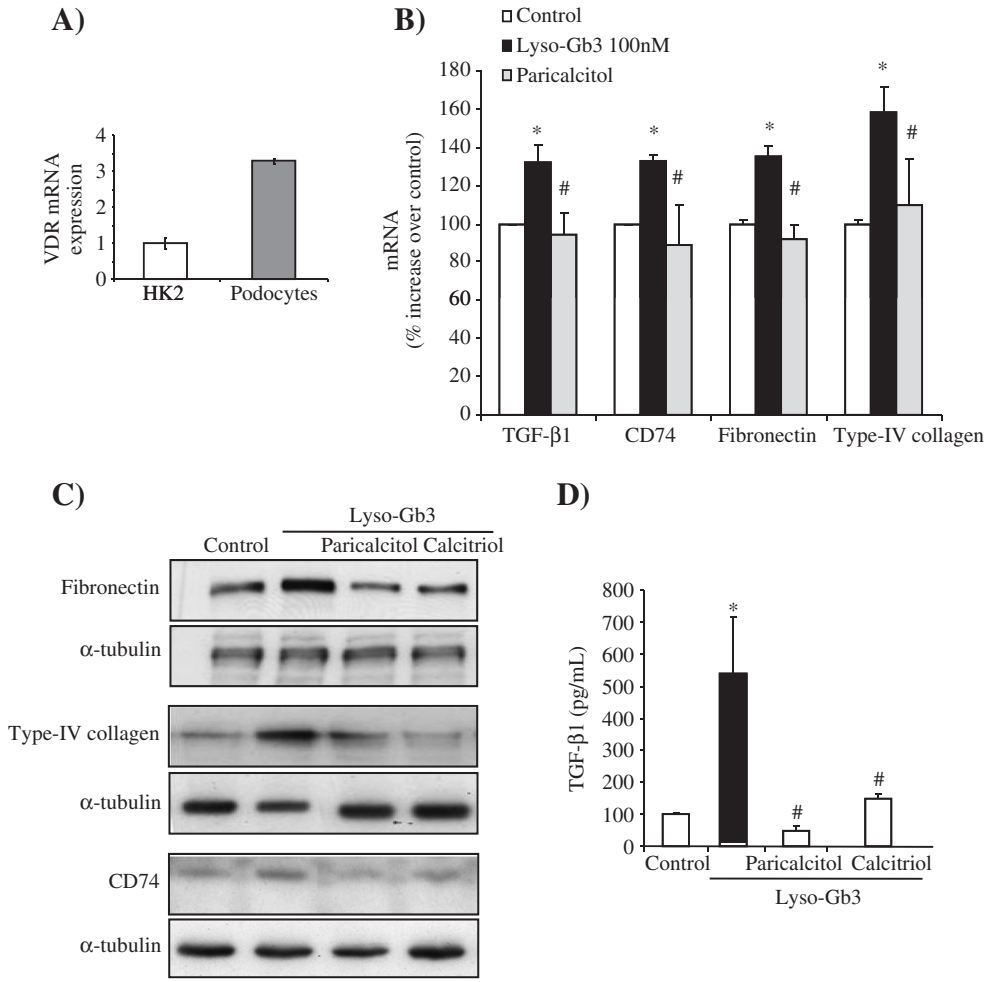

Fig. 4. VDR activation inhibits the podocyte response to lyso-Gb3. (**A**) VDR mRNA expression confirmed by qRT–PCR in cultured podocytes. For comparison, VDR mRNA levels in human proximal tubular HK2 cells are presented, and results are expressed as fold-difference over HK2 levels. (**B**–**D**) Human podocytes were pre-treated with paricalcitol or calcitriol for 1 h before stimulation with 100 nM lyso-Gb3 for 24 h. (**B**) Paricalcitol prevents lyso-Gb3-induced increase in CD74, TGF-beta, fibronectin and type IV collagen mRNA (*P<0.04 vs. control; #P<0.04 vs. lyso-Gb3 alone). Mean±SD of 3–4 experiments. (**C**) Both paricalcitol and calcitriol prevent the podocyte response to lyso-Gb3 at the protein level. Representative western blot. (**D**) Both paricalcitol and calcitriol prevent the increase in supernatant TGF-β1 protein induced by lyso-Gb3. ELISA at 24 h. *P<0.005 vs. control; #P<0.005 vs. lyso-Gb3 alone.

ERT in improving outcomes. Glomerulosclerosis is characterized by podocyte injury and loss and glomerular accumulation of ECM, and podocyte injury causes proteinuria [18,28]. In this regard, ACEIs/ARBs were used in Fabry disease based upon their efficacy in DN, another metabolic proteinuric kidney disease with podocyte injury. Addition of ACEIs/ARBs to ERT has successfully lowered proteinuria in small trials in Fabry nephropathy [14]. However, Fabry patients may be at excess risk for hypotension, and these drugs may not be used in some patients. Thus, additional adjuvant antiproteinuric drugs are required. A correct understanding of the molecular pathway leading to podocyte injury in Fabry disease would increase the spectrum of adjuvant therapeutic options. Lyso-Gb3 is a biologically active cationic amphiphile with a large polar sugar moiety, rendering it relatively hydrophilic and water soluble. ERT can reduce, but not easily normalize, plasma lyso-Gb3 [17]. In podocytes, this metabolite engaged secondary mediators of podocyte injury, such as TGF-β1 and CD74. This reminds of the observation that, in podocytes, high glucose increases TGF-β1, a critical mediator of ECM production, fibrosis and podocyte injury [29–33], and CD74, a MIF receptor that regulates the expression of lethal cytokines [20]. The finding, for the first time, that a metabolite accumulated in Fabry disease modulates the synthesis of mediators involved in DN glomerular injury further supports the notion that we may learn from DN in order to advance in the understanding of Fabry disease. In this regard, paricalcitol, a selective VDR activator that reduces proteinuria in DN [15,16], also interrupted the injurious pathway activated by lyso-Gb3 in podocytes. The natural VDR activator calcitriol had a similar protective effect. It is interesting to note that patients with chronic kidney disease frequently have deficiencies of both 25(OH) vitamin D and calcitriol [34,35]. In this regard, Fabry patients with kidney disease should follow current recommendations regarding identification and treatment of vitamin D deficiency [34]. We cannot yet recommend the use of VDR activators for the purpose of nephroprotection based on cell culture studies. However, Fabry patients with more ad-

vanced kidney injury, those in which ERT may be less beneficial, may have an indication for VDR activator therapy because of vitamin D deficiency or secondary hyperparathyroidism [34,35]. A potential nephroprotective effect of paricalcitol or calcitriol should be monitored in Fabry patients treated with these drugs because of secondary hyperparathyroidism, in order to get further insights into the clinical relevance of our observation. Confirmation of the antiproteinuric effect of paricalcitol in recent clinical trials may expand the potential indication in Fabry disease to earlier stages of nephropathy.

In summary, we have identified a novel role for lyso-Gb3 in glomerular injury and characterized, for the first time, a molecular pathway of potential pathogenic significance in podocyte injury in Fabry nephropathy. Interestingly, this pathway may be regulated by currently marketed drugs with antiproteinuric potential, such as VDR activators.

Acknowledgements. We acknowledge the following grant supports: FEEL (Fundacion Española para el estudio de enfermedades lisosomales), FIS 06/0046, FIS PS09/00447 PI081564, EUproject DIALOK: LSHB-CT-2007-036644, ISCIII-RETIC REDinREN/RD06/0016, Comunidad de Madrid/FRACM/S-BIO0283/2006, SAF 2007/63648, and CAM S-GEN-0247/2006; and the following salary supports: MEC to M.D.S.N., FIS to M.D.S.N. and A.B.S., and Programa Intensificación Actividad Investigadora (ISCIII/Agencia Laín-Entralgo/CM) to A.O.

Conflict of interest statement. A.O. is a consultant for Genzyme.

References

1. Brady RO, Gal AE, Bradley RM *et al*. Enzymatic defect in Fabry's disease ceramidetrihexosidase deficiency. *N Engl J Med* 1967; 276: 1163–1167
2. Kint JA. Fabry's disease: alpha-galactosidase deficiency. *Science* 1970; 167: 1268–1269
3. Askari H, Kaneski CR, Semino-Mora C *et al*. Cellular and tissue localization of globotriaosylceramide in Fabry disease. *Virchows Arch* 2007; 451: 823–834
4. Ortiz A, Oliveira JP, Wanner C *et al*. Recommendations and guidelines for the diagnosis and treatment of Fabry nephropathy in adults. *Nat Clin Pract Nephrol* 2008; 4: 327–336
5. Waldek S, Patel MR, Banikazemi M *et al*. Life expectancy and cause of death in males and females with Fabry disease: findings from the Fabry Registry. *Genet Med* 2009; 11: 790–796
6. Alroy J, Sabnis S, Kopp JB. Renal pathology in Fabry disease. *J Am Soc Nephrol* 2002; 13: S134–S138
7. Fogo AB, Bostad L, Svarstad E *et al*. Scoring system for renal pathology in Fabry disease: report of the International Study Group of Fabry Nephropathy (ISGFN). *Nephrol Dial Transplant* 2010; 25: 2168–2177
8. Ortiz A, Oliveira JP, Waldek S *et al*. Nephropathy in males and females with Fabry disease: cross-sectional description of patients before treatment with enzyme replacement therapy. *Nephrol Dial Transplant* 2008; 23: 1600–1607
9. Ortiz A, Cianciaruso B, Cizmarik M *et al*. End-stage renal disease in patients with Fabry disease: natural history data from the Fabry Registry. *Nephrol Dial Transplant* 2010; 25: 769–775
10. Branton MH, Schiffmann R, Sabnis SG *et al*. Natural history of Fabry renal disease: influence of alpha-galactosidase A activity and genetic mutations on clinical course. *Medicine (Baltimore)* 2002; 81: 122–138
11. Mora C, Navarro JF. Inflammation and diabetic nephropathy. *Curr Diab Rep* 2006; 6: 463–468
12. Germain DP, Waldek S, Banikazemi M *et al*. Sustained, long-term renal stabilization after 54 months of agalsidase beta therapy in patients with Fabry disease. *J Am Soc Nephrol* 2007; 18: 1547–1557
13. Torra R. Renal manifestations in Fabry disease and therapeutic options. *Kidney Int Suppl* 2008; 111: S29–S32
14. Tahir H, Jackson LL, Warnock DG. Antiproteinuric therapy and Fabry nephropathy: sustained reduction of proteinuria in patients receiving enzyme replacement therapy with agalsidase-beta. *J Am Soc Nephrol* 2007; 18: 2609–2617
15. Agarwal R, Acharya M, Tian J *et al*. Antiproteinuric effect of oral paricalcitol in chronic kidney disease. *Kidney Int* 2005; 68: 2823–2828
16. Lambers Heerspink HJ, Agarwal R, Coyne DW *et al*. The selective vitamin D receptor activator for albuminuria lowering (VITAL) study: study design and baseline characteristics. *Am J Nephrol* 2009; 30: 280–286
17. Aerts JM, Groener JE, Kuiper S *et al*. Elevated globotriaosylsphingosine is a hallmark of Fabry disease. *Proc Natl Acad Sci U S A* 2008; 105: 2812–2817
18. Moreno JA, Sanchez-Nino MD, Sanz AB *et al*. A slit in podocyte death. *Curr Med Chem* 2008; 15: 1645–1654
19. Ziyadeh FN. Mediators of diabetic renal disease: the case for TGF-beta as the major mediator. *J Am Soc Nephrol* 2004; 15: S55–S57
20. Sanchez-Nino MD, Sanz AB, Ihalmo P *et al*. The MIF receptor CD74 in diabetic podocyte injury. *J Am Soc Nephrol* 2009; 20: 353–362
21. Saleem MA, O'Hare MJ, Reiser J *et al*. A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol* 2002; 13: 630–638
22. Carvajal G, Rodriguez-Vita J, Rodrigues-Diez R *et al*. Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation. *Kidney Int* 2008; 74: 585–595
23. Cardus A, Panizo S, Parisi E *et al*. Differential effects of vitamin D analogs on vascular calcification. *J Bone Miner Res* 2007; 22: 860–866
24. Ruiz-Ortega M, Ruperez M, Esteban V *et al*. Angiotensin II: a key factor in the inflammatory and fibrotic response in kidney diseases. *Nephrol Dial Transplant* 2006; 21: 16–20
25. Iglesias de la Cruz MC, Ziyadeh FN, Isono M *et al*. Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes. *Kidney Int* 2002; 62: 901–913
26. Mori Y, Ishida W, Bhattacharyya S *et al*. Selective inhibition of activin receptor-like kinase 5 signaling blocks profibrotic transforming growth factor beta responses in skin fibroblasts. *Arthritis Rheum* 2004; 50: 4008–4021
27. Yamauchi K, Takano Y, Kasai A *et al*. Screening and identification of substances that regulate nephrin gene expression using engineered reporter podocytes. *Kidney Int* 2006; 70: 892–900
28. Wiggins RC. The spectrum of podocytopathies: a unifying view of glomerular diseases. *Kidney Int* 2007; 71: 1205–1214
29. Park IS, Kiyomoto H, Abboud SL *et al*. Expression of transforming growth factor-beta and type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 1997; 46: 473–480
30. Sharma K, Ziyadeh FN, Alzahabi B *et al*. Increased renal production of transforming growth factor-beta1 in patients with type II diabetes. *Diabetes* 1997; 46: 854–859
31. Pantsulaia T. Role of TGF-beta in pathogenesis of diabetic nephropathy. *Georgian Med News* 2006; 131: 13–18
32. Alsaad KO, Herzenberg AM. Distinguishing diabetic nephropathy from other causes of glomerulosclerosis: an update. *J Clin Pathol* 2007; 60: 18–26
33. Mason RM, Wahab NA. Extracellular matrix metabolism in diabetic nephropathy. *J Am Soc Nephrol* 2003; 14: 1358–1373
34. Kidney Disease: Improving Global Outcomes (KDIGO) CKD-MBD Work Group. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009; 113: S1–S130
35. K/DOQI clinical practice guidelines for bone metabolism and disease in chronic kidney disease. *Am J Kidney Dis* 2003; 42: S1–S201

Received for publication: 11.1.10; Accepted in revised form: 11.5.10