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Over-expression of muscle glycogen synthase in human diabetic nephropathy

Rodrigo Gatica · Romina Bertinat · Pamela Silva · Pamela Kairath · Felipe Slebe · Fabián Pardo · María J. Ramírez · Juan C. Slebe · José M. Campistol · Francisco Nualart · Carme Caelles · Alejandro J. Yáñez

Abstract Diabetic nephropathy (DN) is a major complication of diabetic patients and the leading cause of end-stage renal disease. Glomerular dysfunction plays a critical role in DN, but deterioration of renal function also correlates with tubular alterations. Human DN is characterized by glycogen accumulation in tubules. Although this pathological feature has long been recognized, little information exists about the triggering mechanism. In this study, we detected over-expression of muscle glycogen synthase (MGS) in diabetic human kidney. This enhanced expression suggests the participation of MGS in renal metabolic changes associated with diabetes. HK2 human renal cell line exhibited an intrinsic ability to synthesize glycogen, which was enhanced after over-expression of protein targeting to glycogen. A correlation between increased glycogen amount and cell death was observed. Based on a previous transcriptome study on human diabetic kidney disease, significant differences in the expression of genes involved in glycogen metabolism were analyzed. We propose that glucose, but not insulin, is the main modulator of MGS activity in HK2 cells, suggesting that blood glucose control is the best approach to modulate renal glycogen-induced damage during long-term diabetes.

Keywords Glycogen · Glycogen synthase · Diabetic nephropathy · Human kidney

Introduction

Diabetic nephropathy (DN) is one of the major complications of diabetic patients and is the leading cause of end-stage renal disease, affecting one-third of patients with either type 1 or 2 diabetes mellitus (Reutens and Atkins 2011). Although glomerular dysfunction has an important

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R. Gatica
Facultad de Medicina, Universidad San Sebastián, Puerto Montt, Chile

R. Bertinat · P. Silva · P. Kairath · J. C. Slebe · A. J. Yáñez (✉)
Instituto de Bioquímica y Microbiología, Universidad Austral de Chile, Isla Teja S/N, Valdivia, Chile
e-mail: ayanez@uach.cl

F. Slebe
Institute for Research in Biomedicine (IRB), University of Barcelona, Barcelona, Spain

F. Pardo
Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

M. J. Ramírez · J. M. Campistol
Department of Nephrology and Renal Transplantation, Hospital Clinic, Barcelona, Spain

F. Nualart · A. J. Yáñez
Centro de Microscopía Avanzada, CMA-Bío Bío, Universidad de Concepción, Concepción, Chile

C. Caelles
Universidad de Barcelona, Barcelona, Spain

role in establishment and progression of DN, deterioration of renal function also correlates with tubular alterations (Singh et al. 2008). An early observation in human DN biopsies has shown that glycogen is accumulated in tubules (Armanni 1877; Ritchie and Waugh 1957). Indeed, deposition of glycogen in other renal pathologies suggests that this glucose polymer may be toxic for renal cell function (Cammisotto et al. 2008). The exact mechanism by which glycogen accumulation occurs is not yet known.

Glucose uptake in the kidney occurs in an insulin-independent manner; hence, if more glucose is filtered by the glomerulus, more glucose is reabsorbed and returned to circulation by the proximal tubule, setting a vicious circle (Vallon 2011). Nevertheless, when the proximal tubule capacity is overwhelmed, glucose becomes available for downstream nephron segments that are not normally exposed to the sugar from the luminal face (DeFronzo et al. 2012). Under normal conditions, the human kidney does not contain significant glycogen stores, and ultrastructural studies have shown small amounts of intra-cytoplasmic glycogen within cells of the middle portion of the proximal tubule (Biava et al. 1966). These are the only available data regarding the ability of human proximal tubules to accumulate glycogen. However, human diabetic patients present a characteristic renal histopathological feature, the so-called Armanni-Ebstein lesion, resulting from extensive glycogen deposition in the tubular cells (Armanni 1877; Ritchie and Waugh 1957; Silva 2005). Therefore, the increased renal glucose uptake observed in diabetic subjects (Meyer et al. 2004) may influence the glycogen accumulation. The healthy rat kidney cannot synthesize appreciable amounts of glycogen either (Nannipieri et al. 2001; Wirthensohn and Guder 1986), but diabetes induced a 30-fold increase in glycogen levels and also a significant increase in total glycogen synthase activity (Khandelwal et al. 1979). Other studies have determined that glycogen granules are accumulated in both proximal and distal tubules from diabetic rat kidney (Kang et al. 2005).

Glycogen is a branched polymer of glucose which serves as a reservoir in the normal state (Young 1957). Once inside the cell, glucose metabolism is initiated by its phosphorylation into glucose 6-phosphate (G6P). G6P is converted into G1P and then into UDP-glucose, and synthesis of glycogen is catalyzed by one of two different isoforms of the enzyme glycogen synthase (GS): liver GS (LGS) or muscle GS (MGS, Ferrer et al. 2003). LGS expression is specific for the liver, whereas MGS is expressed in almost all tissues; but both isoforms are regulated by G6P allosteric activation and by inactivating phosphorylation (Ferrer et al. 2003; Ros et al. 2009). Fully phosphorylated GS is considered to be inactive, whereas dephosphorylated GS is active. GS activation by dephosphorylation requires glycogen-targeting subunits of phosphatases, such as protein targeting to glycogen (PTG, Graham 2009), and distinct phosphorylated forms of GS in combination with G6P determine different degrees of activation (Prats et al. 2009; Ros et al. 2009). Insulin is the main glycogenic hormone in liver and muscle, stimulating G6P production and inhibiting glycogen synthase kinase-3 (GSK-3), which is one of the most important kinase activities involved in GS phosphorylation (MacAulay et al. 2005).

Renal expression of different GS isozymes in rats has been previously determined. MGS, but not LGS, has been detected in kidney extracts from non-diabetic rats (Kaslow and Lesikar 1984; Kaslow et al. 1985). On the basis of these findings, we proposed to analyze the expression of MGS in renal cortex from human type 2 diabetic patients, in parallel with histological analysis of glycogen deposition. In this study, which is an extension of previous work in rodent models, we detected over-expression of MGS in the diabetic human kidney and a correlation between increased glycogen amounts and cell death in a model of human renal tubule cells. Analysis of previous transcriptomic studies in human kidney (Woroniecka et al. 2011) demonstrated that diabetic tubuli present altered expression of genes involved in glycogen metabolism that, together with enhanced glucose uptake, may explain pathological glycogen accumulation in the diabetic kidney. Our results propose that glucose, but not insulin, is the main modulator of MGS activity in a renal cell line, suggesting that blood glucose control is the best way to modulate renal glycogen-induced damage during diabetes.

Materials and methods

Human samples

Samples were obtained from the non-cancerous pole of surgically removed kidney from adult non-diabetic ($n = 4$) and type 2 diabetic ($n = 4$) patients with small (<6 cm diameter) renal carcinoma, at the Urology Department of Hospital Clinic, Barcelona, Spain. The institutional review board at Hospital Clinic approved the study, and patients gave informed consent for biopsy collection and analysis. Diabetic patients were selected by fasting glycemia >140 mg/dl and glycosylated hemoglobin (HbA1c) >6.5 %. Average age of non-diabetic patients was 69 ± 8 years old ($n = 4$; all male patients), and that of diabetic patients was 67 ± 8 years old ($n = 4$; three male and one female patients). These eight samples were processed for qPCR, Western blot, and histochemical analysis. Besides, two more control samples were available only for qPCR analysis.

Antibodies

Rabbit anti-liver/muscle GS (dilution 1:5,000; 15B1 #3,886), rabbit anti-phospho-GS Ser641 (dilution 1:2,000;

#3,891), and rabbit anti-β tubulin (dilution 1:2,000; #2,146) were from Cell Signaling Technologies (Beverly, MA). Rabbit anti-rat LGS (dilution 1:10,000) was prepared in Dr. Guinovart's laboratory (Ros et al. 2009). Rabbit anti-hepatic FBPase (proximal tubule marker; dilution 1:1,000) was prepared in our laboratory (Gatica et al. 2013; Yáñez et al. 2005). Mouse monoclonal antibody against glycogen has been previously used (dilution 1:500; Baba 1993; Ros et al. 2009). Negative controls were incubated with 22 U/ml α-amylase (Sigma #10,065, St. Louis, MO) in PBS for 30 min at 37 °C, previous to incubation with anti-glycogen antibody. Secondary antibodies were HRP-conjugated donkey anti-rabbit IgG (dilution 1:5,000; Jackson Immuno Research, West Grove, PA), Alexa Fluor 633-conjugated goat anti-rabbit IgG, and Alexa Fluor 488-conjugated goat anti-mouse IgG/IgM (dilution 1:500; Molecular probes, Eugene, OR).

Histochemical analysis

Pieces of non-diabetic and diabetic human kidney were fixed in formalin and embedded in paraffin, as routinely processed in the clinic. Immunofluorescence studies were conducted as previously described by Gatica et al. (2013). Briefly, tissue was deparaffinized in xylene, rehydrated in graded ethanol, blocked with 3 % BSA and permeabilized with 0.3 % Triton X-100 for 20 min. Primary antibody was incubated overnight at 4 °C. Alexa Fluor-secondary antibodies were used, and counter staining was carried out with DAPI. For periodic acid–schiff (PAS) staining, we used the PAS Staining Kit from Merck-Millipore (Billerica, MA), following the manufacturer's instructions.

Cell culture

Human proximal tubule cell line, HK-2, was obtained from the ATCC and cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5 mM glucose, 1 mM glutamine and 5 % fetal bovine serum and antibiotics. For infection, HK-2 cells were incubated for 2 h with green fluorescent protein (GFP) or protein targeting to glycogen (PTG) adenoviruses, both under the control of CMV promoter (Vilchez et al. 2007), at 10, 20, 40 and 80 multiplicities of infection (MOI). After removal of the virus-containing media, infected cells were maintained for 48 h in culture medium to allow protein over-expression. DMEM without serum was used as basal medium for experiments. Glycogen levels were determined as previously described by Vilchez et al. (2007). Briefly, cells were homogenized with 30 % KOH and heated for 15 min at 100 °C. Aliquots were spread on Whatman 3M filter paper and washed in cold 66 % ethanol. Finally, glycogen was digested and glucose content determined by

an enzyme-coupled assay. Cell viability was assessed by trypan blue exclusion.

Western blot

Cell homogenates were prepared with lysis buffer (20 mM HEPES pH 7.5, 10 mM EGTA, 2.5 mM MgC_{12}) plus 1 % NP-40 and protease inhibitors (Calbiochem, Darmstadt, Germany). Twenty micrograms of total proteins were fractionated in 4–12 % SDS-PAGE, transferred to PVDF membranes and probed overnight with primary antibodies. Following incubation with a HRP-conjugated secondary antibody, reaction was developed using the Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL).

Real-time RT-PCR

Total RNA was extracted from human renal cortex using SV total RNA isolation system (Promega, Madison, WI). cDNA was synthesized from 2 μg total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA). For qPCR, we used unlabeled specific primers for 18S (reference gene, Hs03003631_g1) and muscle GS gene (Hs00157863_m1) and used TaqMan Universal PCR Master Mix No AmpErase UNG and TaqMan ABI7700 sequence Detection System (all from Applied Biosystems, Foster City, CA). Primers were annealed at 60 °C and run for 40 cycles. Data were captured using Sequence Detector Software 2.2 (Applied Biosystems, Foster City, CA).

Analysis of transcriptomic data

High-throughput transcriptome analysis of human diabetic kidney disease (DKD Tubuli vs. Control Tubuli) was obtained from the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI), with the accession number GSE30529. Basically, values for clustering were obtained after standardizing the gene values. The mean value of each gene was subtracted from the value of this gene in each array and was divided by the standard deviation of this gene in all the arrays. Clustering was performed with a distance metric of 1 − correlation, centroid linkage method and gene ordering by cluster tightness. Data genes were compared to the same probe set ID for each gene according to the manufacturer (Affymatrix).

Statistical analysis

All experiments were performed at least three times, and results are expressed as the mean ± SE. Statistical analysis

was performed with Student's *t* test. Differences were considered statistically significant for $p < 0.05$.

Results

Histological analysis of renal biopsies and MGS expression

PAS staining of biopsies from diabetic patients (patients 5–8; Fig. 1i–p) demonstrated expected alterations in renal morphology (thickening of glomerular and tubular basal membranes, glomerular sclerosis, inflammatory infiltration, tubular atrophy), compared with the non-diabetic controls (Patients 1–4; Fig. 1a–h). qPCR analysis showed a significant increase in MGS mRNA expression in diabetic vs non-diabetic renal cortex (Fig. 1q). Moreover, protein levels of GS were elevated (at different degrees) in all diabetic biopsies analyzed (Fig. 1r). These clear differences in expression of MGS between non-diabetic and diabetic kidneys were consistent in this analysis, despite the fact that four specimens per group are low in number. However, it was difficult to obtain enough viable samples from human tissue for the study, especially with renal histology of DN. LGS expression was not detected by Western blot (data not shown). GS electrophoretic mobility is greatly affected by phosphorylation, and the more phosphorylated the higher molecular size in a SDS-PAGE. However, no evident shift on GS electrophoretic mobility was determined in human diabetic renal cortex (Fig. 1r), and an antibody against phospho-GS Ser641 failed to detect GS in human samples (data not shown). These data suggest that GS over-expression is a common phenomenon in human diabetic kidney. By means of a specific monoclonal antibody against glycogen, we observed its deposition in tubules from diabetic, but not control renal cortex biopsies (Fig. 2). Specifically, glycogen was detected in proximal (Fig. 2d, e) and non-proximal tubules (Fig. 2c, d), as indicated by the use of the proximal tubule marker, fructose 1,6-bisphosphatase (FBPase). Moreover, one sample showed a negligible glycogen signal (Fig. 2b). Overall, a clear heterogeneity in glycogen deposition was observed between diabetic biopsies. As shown in Fig. 1, there were varying degrees of pathology occurring among the specimens. Some have very prominent inflammatory infiltrations with interstitial fibrosis and others have moderate interstitial fibrosis with little to no inflammatory infiltrate. Interestingly, glycogen was detected in biopsies from diabetic patients with fewer renal morphological alterations (patients 6–8, Figs. 1j–l, n–p; 2c–e), whereas those with higher degree of fibrosis and inflammation (Patient 5, Fig. 1i and m) did not reveal the presence of glycogen (Fig. 2b). Nevertheless, correlation between glycogen deposition and the degree of inflammation could not be confirmed due to the low number of samples.

Glycogen accumulation in human renal proximal tubule cell line

Hyperglycemia and hyperinsulinemia are hallmarks of diabetes, and renal proximal tubules are highly exposed to this detrimental altered environment. We used the human HK-2 renal proximal tubule cell line to determine the separate effects of these two stimuli on GS phosphorylation. HK2 cells were cultured in serum-enriched medium (see "Materials and methods"). The use of an antibody that recognizes total GS (phosphorylated and unphosphorylated forms) enabled us to discriminate between different forms of the enzyme via their differential migration in SDS-PAGE. Culture in the same media with additional supplementation of 30 mM glucose and 10 nM insulin allowed us to detect two forms of GS, one of approximately 85 KDa and other of approximately 80 KDa (Fig. 3, lane 1). Incubation of cells in complete medium in the absence of insulin, glucose or any other substrates led to total loss of 80 KDa band detection (Fig. 3, lane 2), whereas high glucose supplementation alone increased its amount (Fig. 3, lane 3). Insulin alone did not show any effect on detection of the 80 KDa band, but glucose plus insulin did enhance this form, although it did not reach statistical significance (Fig. 3, lanes 4–5, respectively). Although some other factor(s) in the complete medium further stimulated GS migration, glucose alone was enough to induce migration of the 80 KDa band of GS. Hence, in these conditions, glucose was the main inducer of changes in the migration pattern of GS, which could be related to activation of the enzyme. Unfortunately, the antibody against P-GS Ser641 was unable to detect GS in HK-2 cells (data not shown). Once we determined that HK-2 cells expressed GS (Fig. 3), we studied the capacity of this human cell line to synthesize and store glycogen. Full activation of GS requires complete dephosphorylation, and a glycogen-targeting subunit of protein phosphatase is needed. We decided to study PTG over-expression on glycogen accumulation, as it has been proven to work in other cell models (refer to Vilchez et al. 2007; Villarroel-Espíndola et al. 2013). We used adenovirus-mediated over-expression of GFP (control of infection) and PTG at different MOIs and determined the dose–response effect after 48 h. Medium was replaced 24 h and again 3 h before the experiment with DMEM supplemented with 30 mM glucose, and finally, cells were processed to determine glycogen levels and cell viability. Non-infected cells were able to synthesize a significant amount of glycogen after stimulation with glucose (Fig. 4a, white bars). As expected, any further increase in glycogen storage was observed after infection with GFP adenovirus (Fig. 4a, light gray bars). In contrast, a dose-dependent accumulation of glycogen was determined after infection with increasing MOIs of
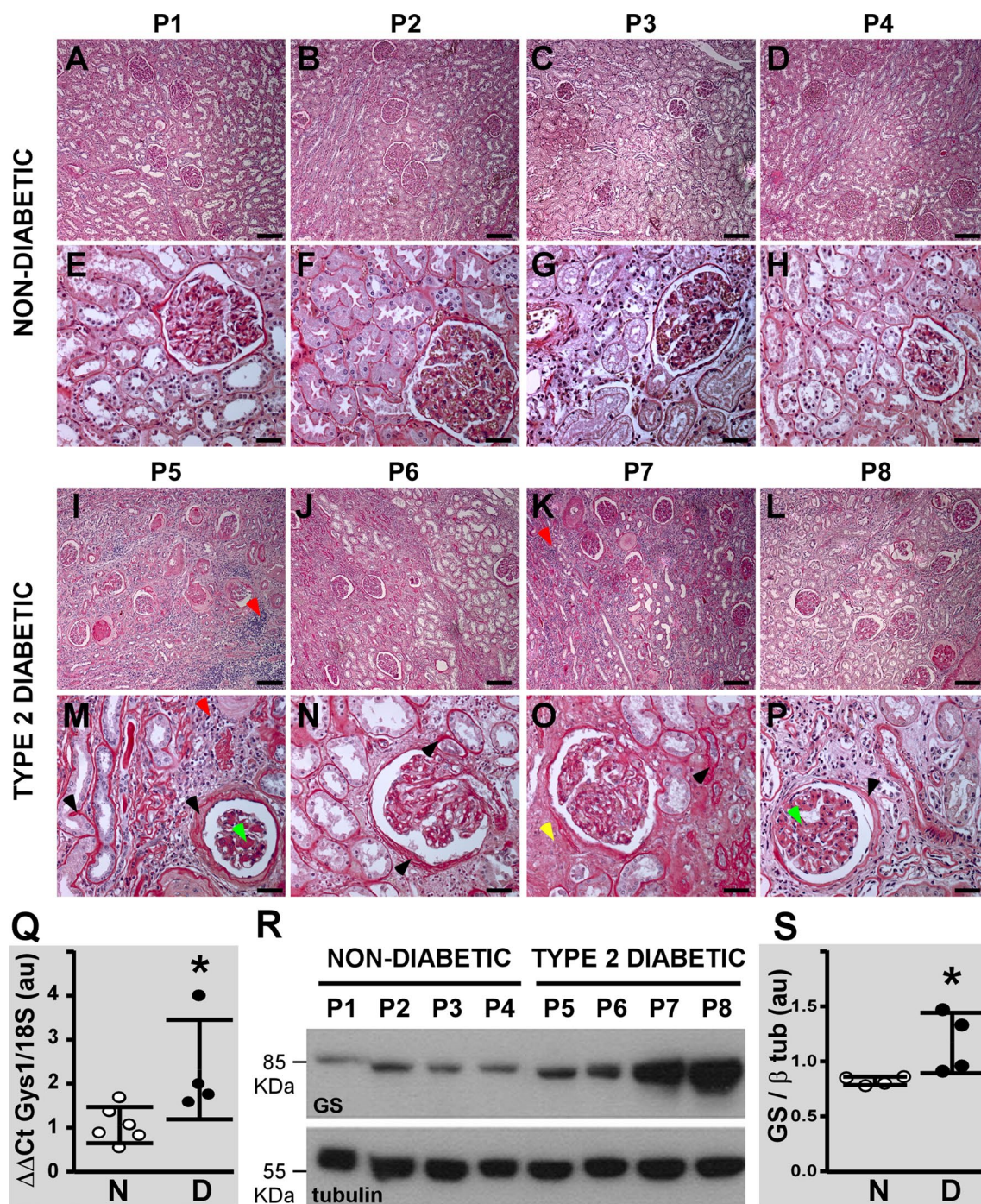
Fig. 1 MGS expression in biopsies from DN patients. PAS staining was carried out in human renal biopsies fixed in formalin and embedded in paraffin (**a–p**). Representative images of all biopsies from non-diabetic (P1–4) and type 2 diabetic (P5–8) patients are shown at lower (**a–d**, **i–l** *scale bar* 500 µm) and higher (**e–h**, **m–p** *scale bar* 100 µm) magnification. Characteristic morphological alterations of DN are shown: thickening of glomerular and tubular basal membrane (*black arrowhead*), inflammatory infiltrate (*red arrowhead*), inter-stitial fibrosis (*yellow arrowhead*), and glomerular sclerosis (*green arrowhead*). qPCR analysis using TaqMan-specific primers against muscle glycogen synthase (MGS) (**Q**) and Western blot analysis against total GS (**R**) (normalized and semi-quantified against tubulin; **S**) were performed in renal biopsies from non-diabetic (*white circles*) and type 2 diabetic (*black circles*) patients. $n = 4$ for each group, except $n = 6$ control samples for qPCR (**Q**). *$p < 0.05$

Fig. 2 Immunohistochemical detection of glycogen in biopsies from DN patients. Human renal biopsies, fixed in formalin and embedded in paraffin, were analyzed by means of immunofluorescence using a monoclonal antibody against glycogen (*red channel*) and a polyclonal antibody against a proximal tubule marker (FBPase, *green channel*). Dapi was used as the nuclear counterstaining (*blue channel*). Panel **A** is representative of all four non-diabetic biopsies (Patients P1–4), and **b–e** correspond to diabetic biopsies (Patients P5–8). *Scale bar* 30 µm
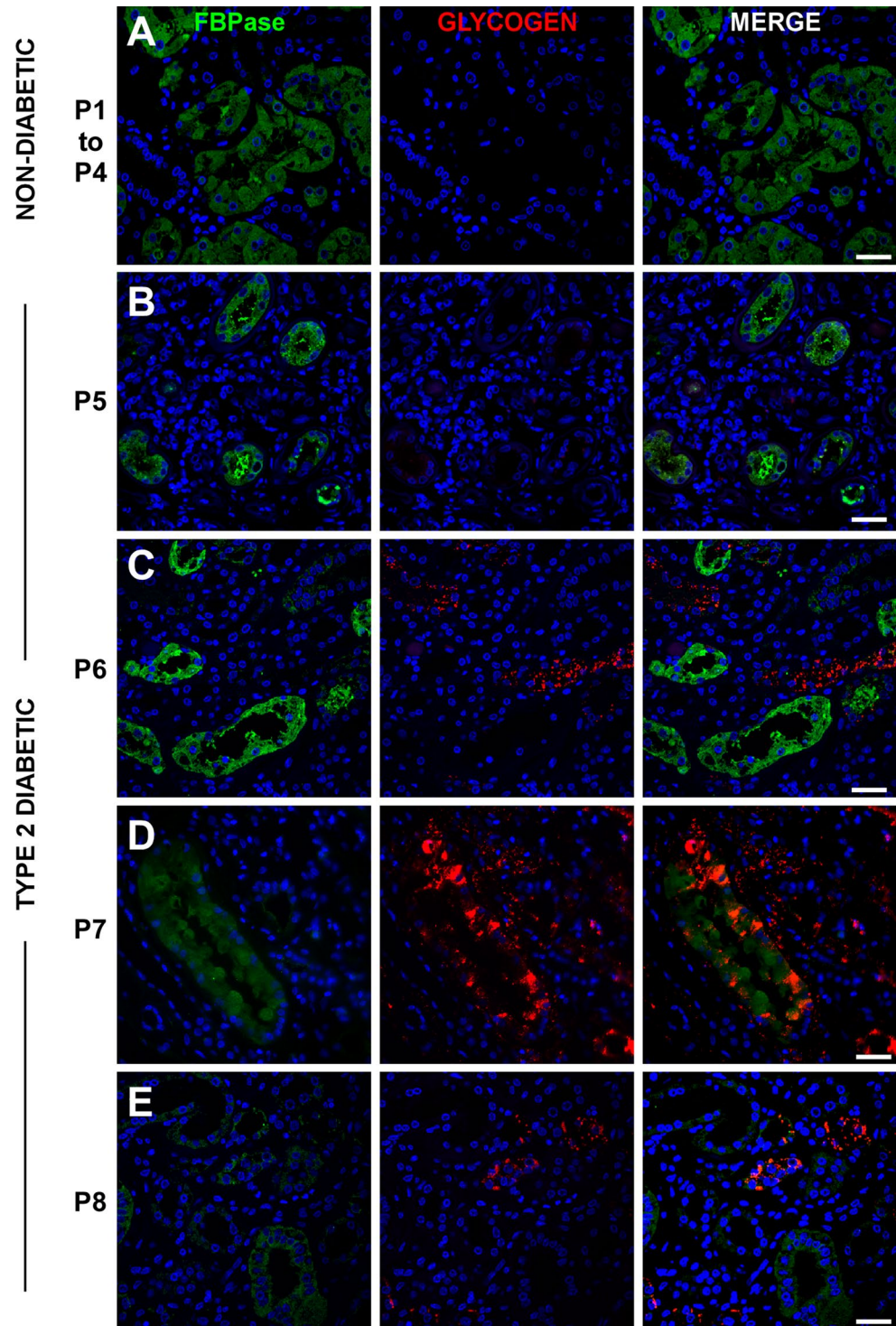
PTG adenovirus (Fig. 4a, dark gray bars). In parallel, analysis of cell viability after infection with GFP or PTG adenovirus was performed, revealing that over-expression of GFP did not significantly induce cell death, whereas PTG over-expression promoted more than 50 % decrease in cell viability, that was evident even at the lowest MOI (Fig. 4b–c).
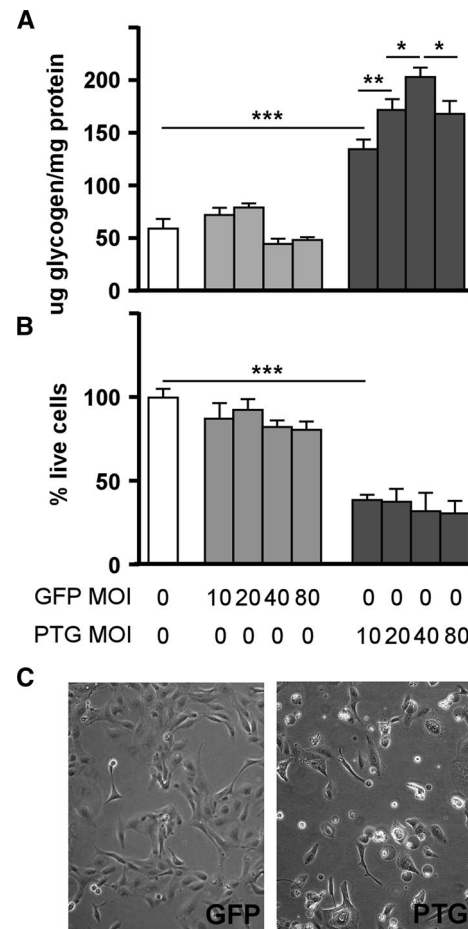
Altered expression of genes involved in glycogen metabolism in human diabetic tubuli

A previous transcriptomic study of laser microdissected tubuli from diabetic and non-diabetic human kidney (Woroniecka et al. 2011; accession number GSE30529 at GEO, NCBI, refer to "Materials and methods") was
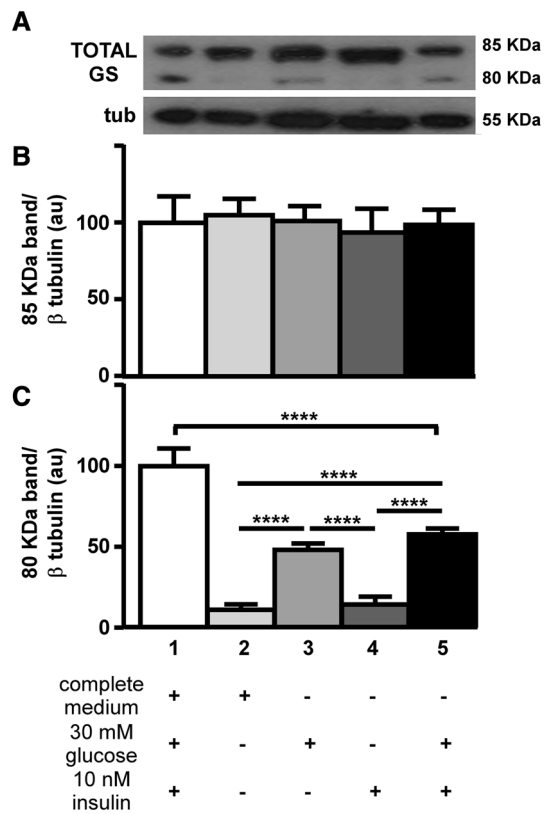
Fig. 3 Effect of glucose and insulin over GS migration pattern in SDS-PAGE. HK-2 cells were cultured in complete (+) or basal (−) DMEM medium in the presence of 30 mM glucose, or 10 nM insulin, or both for 3 h. Total proteins were separated by SDS-PAGE and analyzed by Western blot with anti-total GS antibody (**a**). 85 KDa band **b** and 80 KDa band **c** were semi-quantified and normalized against β tubulin (tub). ****$p < 0.0001$; $n = 5$


Fig. 4 Glycogen accumulation and human renal cell viability. HK-2 cells were infected with different MOIs (10, 20, 40 or 80) of green fluorescent protein (GFP) adenovirus (*light gray bars*), protein targeting to glycogen (PTG) adenovirus (*dark gray bars*), or not infected at all (*white bars*), and cultured for 48 h before glycogen production **a** and cell viability **b** were assessed in the presence of 30 mM glucose. Representative images of GFP- or PTG-infected cells are shown. **c** *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 5$

analyzed to detect changes in expression of genes directly and indirectly related to glycogen metabolism. The analysis revealed no significant changes in the expression of MGS (GYS1), glycogen-debranching enzyme (AGL), glycogen-branching enzyme (GBE1), glycogenin 2 (GYG2), protein phosphatase 1 regulatory subunit 3A (PPP1R3A, also known as GM or PP1G), protein phosphatase 1 regulatory subunit 3D (PPP1R3D, also known as R6), and insulin receptor substrate 1 (IRS1) genes (Fig. 5a–g, respectively, light gray). In contrast, analysis of LGS (GYS2), muscle glycogen phosphorylase (PYGM), insulin receptor (INSR), and insulin receptor substrate 2 (IRS2) gene expression showed a significant decrease (Fig. 5h–k, respectively, darker gray) in diabetic vs non-diabetic tubuli. Interestingly, expression of brain glycogen phosphorylase (PYGB), glycogenin 1 (GYG1), protein phosphatase 1 α-catalytic subunit (PPP1CA), protein phosphatase 1 regulatory subunit 3C (PPP1R3C, also known as PTG), and glycogen synthase kinase 3β (GSK3β) genes showed a statistically significant increase (Fig. 5l–p, respectively, darkest gray).

Overall, analysis of these genes suggests the activation of glycogen metabolism in human diabetic tubuli, despite insulin signaling is depressed.

Discussion

Diabetic nephropathy (DN) has commonly been studied as a glomerular disease, but evidence supports an early involvement in tubular dysfunction (Singh et al. 2008). Human and rat diabetic kidney accumulates pathological levels of glycogen in the tubules. Nonetheless, studies of abnormal glycogen deposition in the diabetic kidney have been addressed only in rats (Bamri-Ezzine et al. 2003; Khandelwal et al. 1979; Nannipieri et al. 2001), and no information is available in human kidney. Different
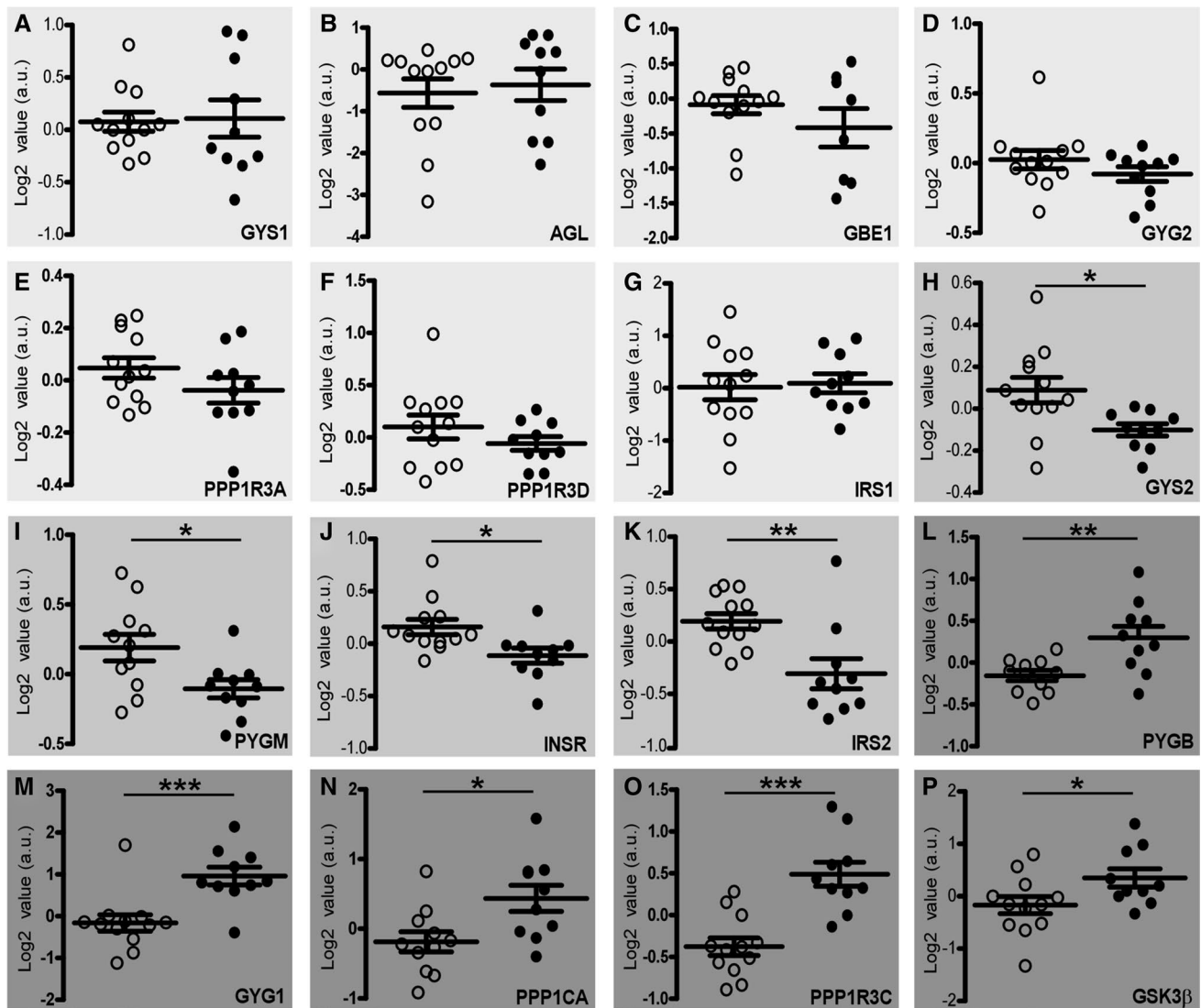
Fig. 5 Transcriptomic analysis of genes involved in glycogen metabolism in human diabetic tubuli. Based on a previously reported transcriptome study (Woroniecka et al. 2011; NCBI-GEO, accession number GSE30529) on human diabetic kidney disease, expression of different genes involved in glycogen metabolism was analyzed between non-diabetic (*open circles*) and diabetic (*full circles*) tubuli. **a** *GYS1* muscle glycogen synthase; **b** *AGL* glycogen-debranching enzyme; **c** *GBE1* glycogen-branching enzyme; **d** *GYG2* glycogenin 2; **e** *PPP1R3A* protein phosphatase 1 regulatory subunit 3A (also known as GM or PP1G); **f** *PPP1R3D* protein phosphatase 1 regulatory subunit 3D (also known as R6); **g** *IRS1* insulin receptor substrate 1; **h** *GYS2* liver glycogen synthase; **i** *PYGM* muscle glycogen phosphorylase; **j** *INSR* insulin receptor; **k** *IRS2* insulin receptor substrate 2; **l** *PYGB* brain glycogen phosphorylase; **m** *GYG1* glycogenin 1; **n** *PPP1CA* protein phosphatase 1 α-catalytic subunit; **o** *PPP1R3C* protein phosphatase 1 regulatory subunit 3C (also known as PTG); **p** *GSK3*β glycogen synthase kinase 3β. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

studies have determined that the predominant location of pathological glycogen accumulation is the thick ascending limb of Henle's loop, whereas less extensive deposition occurs in the distal tubules (Curtis et al. 1947; Holck and Rasch 1993). However, contrasting studies have restricted the lesion to the terminal straight segment of the proximal tubule (Ritchie and Waugh 1957). More recently, studies have also identified glycogen granules in distal and proximal tubules from diabetic rats (Nannipieri et al. 2001; Kang et al. 2005). Kang et al. (2005) have suggested that

diabetes-induced renal damage might occur in the distal tubules first and then extend to the proximal tubules, which is in agreement with the differential ability of each nephron portion to respond to glucose. Indeed, preliminary studies in streptozotocin-diabetic rats led us to detect glycogen accumulation mainly in non-proximal tubules during the first stages of DN, but also in proximal tubules in long-term diabetes (Electronic Supplementary Material 1). Here, we demonstrate for the first time that MGS is also the major GS isoform expressed in human kidney, supporting

previous animal models in rat and rabbit (Kaslow and Lesikar 1984; Kaslow et al. 1985). Moreover, our evidence indicates that renal MGS protein is over-expressed in human DN. G6P is both a precursor of glycogen synthesis and an allosteric activator of GS, and it has been reported to be increased in the diabetic rat kidney (Khandelwal et al. 1979). So MGS over-expression in combination with enhanced glucose uptake and endogenous synthesis of G6P by the diabetic kidney may account for increased glycogen accumulation in this pathological condition.

In non-diabetic conditions, glucose is completely reabsorbed at the first portions of the proximal tubule; hence, activity of glucose transporters in the apical membrane of downstream nephron segments is low (Vallon 2011). Therefore, abnormal luminal glucose exposure and uptake could be the first trigger of pathological induction of glycogen accumulation in renal tubular epithelial cells. Indeed, renal glycogen-positive cells were detected as soon as 1 week after streptozotocin induction of diabetes in rats, when glycemia starts to abnormally rise (Electronic Supplementary Material 2). In contrast, glycogen accumulation decreases in liver and skeletal muscle at the same time (Electronic Supplementary Material 2). These differences can be attributed to insulin-dependent glucose uptake in liver and skeletal muscle, but insulin-independent glucose uptake in the kidney (DeFronzo et al. 2012). Alteration of facilitative glucose transporters expression has been consistently reported in diabetic kidney. In the diabetic rat kidney, the low-affinity/high-capacity GLUT-2, normally exclusively localized to the basolateral membrane, has been localized to the apical membrane of proximal tubules, contributing to the enhanced uptake of glucose from the glomerular ultrafiltrate (Marks et al. 2003). Besides, both GLUT-1 and GLUT-12 are elevated in animal models of DN, with GLUT-12 protein being localized to the apical membrane of distal tubules (Linden et al. 2006), where glycogen accumulation has been detected (Nannipieri et al. 2001; Kang et al. 2005; Electronic Supplementary Material 1 and 2). GLUT-12 functions as a proton-coupled symporter, transporting glucose against its concentration gradient (Wilson-O'Brien et al. 2010). Low urinary pH is a characteristic of human and experimental diabetic models (Bobulescu et al. 2009).

We have previously shown that type 2 diabetic patients and type 1 diabetic rats showed a significant reduction in protein levels and signaling of the insulin receptor in the kidney (Gatica et al. 2013). In contrast, insulin receptor expression was enhanced in the diabetic liver (Gatica et al. 2013), but despite these adaptations, the liver is unable to accumulate glycogen during diabetes (Ros et al. 2011). A key signaling molecule in the insulin pathway is GSK3β, a serine/threonine kinase which is normally active, and whose inactivation by insulin is considered essential for a

normal insulin response. Indeed, several studies have implicated dysregulation of GSK3β in the pathogenesis of diabetes (Sakamaki et al. 2012). Interestingly, activation of GSK3β has been involved in downregulation of MGS and glycogen deposition in rat skeletal muscle cells (MacAulay et al. 2005; Prats et al. 2009). In vivo studies on rabbit MGS showed that most of the phosphate released in response to insulin was removed from sites corresponding to GSK3 phosphorylation (Højlund et al. 2003). Interestingly, our preliminary data showed that diabetes did not induce over-expression of MGS in rat kidney, but a shift from bands of higher to lower molecular weight was detected in SDS-PAGE (Electronic Supplementary (Electronic Supplementary Material 3). However, despite this GS shift, phosphorylation of Ser641 (catalyzed by GSK3β) was constitutively detected (Electronic Supplementary Material 3). This result supports our previous studies demonstrating reduced insulin signaling in diabetic human and rat kidney (Gatica et al. 2013) and the transcriptomic data by Woroniecka et al. (2011), where expression of insulin receptor and IRS2 were decreased while expression of GSK3β was enhanced. However, although phosphorylation leads to inactivation of GS, activity can be restored in the presence of the allosteric activator G6P, which is enhanced in the diabetic kidney (Khandelwal et al. 1979). In our hands, phosphorylation of Ser641 residue in human GS was not detected (data not shown), which may be attributed to a differential phosphorylation of GS in human kidney, or dephosphorylation during sample handling. MGS is also phosphorylated by AMP-activated protein kinase (AMPK; Jørgensen et al. 2004). It has been proposed that one of the metabolic signals stimulating tubular glycogen synthesis in the diabetic kidney is reduced AMPK activity (Cammisotto et al. 2008; Kume et al. 2012). Thus, the complete pathway mediating MGS activation and glycogen accumulation in human DN remains to be elucidated. Moreover, the expression of a novel differently regulated GS isoform in kidney cannot be ruled out.

The accumulation of intracellular glycogen granules (the so-called Armanni-Ebstein lesion) is perhaps the best-known tubular change associated with diabetes in humans. Its pathological significance remains to be established, although it is widely considered to be deleterious. Indeed, the Fanconi–Bickel syndrome, a disorder associated with tubular glycogen accumulation due to a mutation of the GLUT-2 transporter, is also associated with progressive diabetic-like changes (Berry et al. 1995). Furthermore, it has been established that diabetes is associated with an increased risk of renal cell carcinoma and clear cell tubules present excessive storage of glycogen, which has been considered a preneoplastic lesion (Dombrowski et al. 2007). In biopsies from chronic human diabetic patients, PAS staining was not sensitive enough to detect any glycogen, but an antibody against glycogen could reveal its presence.

However, appropriate fixation and processing of tissues for detection of glycogen is still debated in histochemical literature (Trott 1961; Kugler and Wilkinson 1964; Zakout et al. 2010). In most of those studies, the PAS method was used for the detection of glycogen and formalin fixation was found to be inadequate. Moreover, the superiority of using cryosections fixed by Zamboni's fixative (formalin-picric acid) has been demonstrated, even when using the monoclonal antibody against glycogen (Prats et al. 2013). Indeed, rat tissues were fixed in Bouin's fluid for glycogen detection in our study (Electronic Supplementary Material 1 and 2). Thus, the absence of glycogen detection in renal tubules of some patients and the heterogeneity of its distribution among the others could be attributed to inadequate tissue fixation by standard methods in the clinic. Studies by Bamri-Ezzine et al. (2003) have demonstrated that large glycogen accumulations cause apoptosis by a Fas-dependent mechanism in rat kidney. In parallel, Vilchez et al. (2007) have shown that glycogen synthesis is normally absent in neurons and that several neurological diseases exhibit abnormal glycogen accumulation and induction of apoptosis. This pathological accumulation of glycogen has been proposed to be the result of large dephosphorylation of MGS and also stabilization of PTG and MGS proteins (Vilchez et al. 2007). Moreover, it has been recently shown that PTG over-expression in male germ cells also triggered apoptosis (Villarroel-Espíndola et al. 2013). In the present work, we observed that over-expression of PTG was enough to induce large glycogen accumulation and cell death in a human proximal renal cell line. Notably, transcriptomic studies in microdissected tubuli from human diabetic kidney (Woroniecka et al. 2011) showed a significant increase in PTG expression, together with alterations of other key genes involved in glycogen metabolism. However, PTG over-expression may exert other deleterious effects in our cell model, such as sequestering PP1 activity toward the glycogenic machinery and limiting the phosphatase pool for other important cell functions (Bollen 2001). To this end, over-expression of a constitutive active mutant of MGS in neurons from mice and flies produced glycogen accumulation and progressive cell loss (Duran et al. 2012). Since the corresponding experiment of renal MGS over-expression in transgenic animals is not available, we cannot rule out the possibility that glycogen itself is not the detrimental factor, although a correlation between glycogen amount and cell death was determined in our model. Further studies are needed to clarify the mechanism linking diabetes and glycogen accumulation in human DN.

In muscle, glucose metabolism is dependent on insulin signaling (Ferrer et al. 2003). Glucose uptake is decreased in the diabetic muscle, as is mRNA expression of MGS and glycogen content (Vestergaard et al. 1995). In contrast, renal glucose uptake is mostly insulin independent,

so alterations induced by high glucose may contribute to MGS over-expression during DN. Previous studies have shown that hypoxia leads to glycogen accumulation as a metabolic adaptation to prevent or reduce the effects of a more severe hypoxia in muscle (Vigoda et al. 2003). Hypoxia induces a specific gene program under the control of the hypoxia-inducible transcription factor (HIF), which upregulates the expression of MGS gene, correlated with a significant increase in GS activity (Pescador et al. 2010). Normal limitation in renal tissue oxygen supply renders the kidney susceptible to hypoxia (Eckardt et al. 2005). In fact, hyperglycemia results in altered renal oxygen metabolism and decreased renal oxygen tension (Palm 2006; Singh et al. 2008). We can speculate that glycogen synthesis is a mechanism to overcome renal hypoxia, but renal inability to handle excess glycogen could contribute to undesired deleterious effects.

In conclusion, we provide evidence for the first time that diabetes induces over-expression of MGS isoform in human kidney, which may participate in triggering abnormal accumulation of glycogen and cell death. In contrast, glycogen synthesis is impaired in the diabetic liver, and activation of LGS has been shown to restore normal parameters (Ros et al. 2011). GS is considered the rate-limiting enzyme in glycogenesis, but the mechanisms controlling the regulation of GS are still unclear. Hence, a better understanding of the control of glycogen synthesis and degradation between tissues is crucial to provide clues for the development of new anti-diabetic drugs that can normalize overall glycogen metabolism and kidney function.

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