Long-term renal changes in the liver-specific glucokinase knockout mouse: implications for renal disease in MODY2

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Abstract

To investigate the functional and structural renal changes in a long-term liver-specific glucokinase knockout mouse, model of MODY2. Hemizygous glucokinase knockout mice, $gck^{w/-}$ groups, were compared at 6-, 10- and 14-months to their age-matched normal littermates, $gck^{w/w}$ groups. To examine changes we compared body weight, fasting blood glucose, serum insulin and creatinine levels, as well as 24 h urine samples that were collected for urine volume and protein analysis between the two groups. Renal tissues were collected and stained with hemotoxylin-eosin and periodic-acid Schiff, for light microscopic observation. Expression of renal Transforming Growth Factor $\beta 1$ (TGF- $\beta 1$) was determined by Western blot. Our results show that fasting blood glucose levels were significantly higher in $gck^{w/-}$ mice compared to $gck^{w/w}$ mice ($P < 0.01$) for all age groups. Compared to age-matched $gck^{w/w}$ mice, 10-month old $gck^{w/-}$ mice have significantly elevated body weights ($P < 0.01$), and urine volume ($P < 0.05$) and protein concentrations ($P < 0.01$). Renal tubular casts were observed in 10- and 14-month $gck^{w/-}$ mice. Significant increase in mesangial matrix and thickening of the glomerular basement membrane was observed in $gck^{w/-}$ compared to age-matched $gck^{w/w}$ mice at 10 and 14 months. As mice age increases, the levels of renal TGF- $\beta 1$ are observed in both $gck^{w/-}$ and $gck^{w/w}$ mice. Our results indicate that renal changes occur in the liver-specific glucokinase knockout mouse model of MODY2, and suggest that TGF- $\beta 1$ may play a key role in pathogenesis of these renal changes.

Keywords

MODY; Diabetic nephropathy; Animal models

Introduction

Diabetes is the single most frequent cause of end-stage renal disease [1]. Maturity-onset diabetes of the young (MODY) is a clinically heterogeneous group of disorders characterized by nonketotic diabetes mellitus, an autosomal dominant mode of inheritance, an onset usually before the age of 25 years (frequently in childhood or adolescence) and a primary defect in the function of the beta cells of the pancreas [2]. At present, MODY can result from mutations in any one of at least six different genes, one of which encodes the glycolytic enzyme glucokinase (GCK, associated with MODY 2), and the other five encode transcription factors [2]. All of these genes are expressed in beta cells, and mutation of any of them leads to beta-cell dysfunction and diabetes mellitus [3]. These genes are also expressed in other tissues, and abnormalities in liver and kidney function are evident in some forms of MODY [2].

It is generally believed that increased urine albumin excretion in diabetic nephropathy (DN) is mostly glomerular in origin [4]. It has long been appreciated that increased intraglomerular pressure, loss of negatively charged glycosaminoglycans in the basement membrane and, later in the disease process, increased basement membrane pore size all contribute to the albuminuria [4]. Microscopic abnormalities have been described including thickening of the glomerular basement membrane, accumulation of mesangial matrix, and increased numbers of mesangial cells [5]. As disease advances, there is a close relationship between mesangial expansion and declining glomerular filtration [1].

Mesangial expansion also correlates inversely with capillary filtration surface area, which itself correlates to glomerular filtration rate [4]. Recently, in an elegant experimental study, it has been demonstrated that stretching human mesangial cells activates p38 mitogen activated protein kinase via a protein kinase C dependent mechanism, which in turn induces transforming growth factor- β 1 and fibronectin expression [6].

TGF- β has been reported to play a role in experimental glomerulonephritis and human glomerular diseases [7]. High glucose concentration stimulates type IV and type I collagen synthesis in cultured mesangial cells [8]. Treatment with an anti-TGF- β antibody partially reduced proteinuria and renal damage in uninephrectomized rats with overt diabetic nephropathy [9]. In clinical studies, patients with both type 1 and type 2 diabetes have increased renal and glomerular TGF- β expression [10]. Promoter-reporter studies have demonstrated a region in the murine TGF- β 1 promoter that is responsive to high concentrations of glucose. This glucose-responsive promoter region contains an E-box element, an element which has been implicated as a carbohydrate response element in several other glucose-regulated promoters [11]. Using this region as a probe in electrophoretic mobility shift assays, an increased amount of nuclear protein was found to bind the probe from nuclear protein isolated from mesangial cells grown in high glucose compared to low glucose [12].

Most clinical and experimental studies focus on type 1 and type 2 diabetes. Because of the limited number of animal models, there is little data on MODY2. A liver-specific glucokinase knockout mouse has been constructed as a model for MODY2, using the Cre-

loxP gene targeting strategy, and its primary effects have been evaluated [13]. As MODY2 is a dominant gene disease, the hemizygous $gck^{w/-}$ with reduced glucokinase expression should best mimic this disease. Glucokinase knockout mice show increases in blood glucose levels with age and at 6 weeks fasting blood glucose level were significant higher than in $gck^{w/w}$ mice and also displayed impaired glucose tolerance [13].

Since long-term hyperglycemia can result in diabetic complications affecting the eyes, blood vessels, nerves and kidneys [5], the aim of our study was to identify functional and structural renal changes due to long-term reduced liver glucokinase expression in our mouse model, and determine whether this animal model resembles the changes observed in human diabetic kidney disease. If our model is found to accurately reflect human diabetic kidney disease, then, this model could potentially be used to study mechanisms and treatments of renal disease in diabetes.

Materials and methods

Animals

The 'Guidelines for Animal Experiment' approved by the Peking University Health Science Center were followed in these experiments. Liver-specific glucokinase knockout mice were previously generated by our lab [13]. Liver-specific glucokinase knockout mice $gck^{w/-}$ and their littermates of 6, 10, and 14 months were raised by the Department of Laboratory Animal Science, Peking University. Animals were housed three per cage in specific pathogen-free condition with a 12:12 h artificial light cycle. Room temperature and humidity were maintained automatically at about 25° C and 50%, respectively. Animals had free access to sterile standard chow and tap water throughout the experiment.

Assay of biochemistry parameters

Body weight was measured in all mice. An intraperitoneal glucose tolerance test (IPGTT) was performed in each group by i.p. injection of a 20% glucose solution at a dose of $2 \text{ g} \cdot \text{kg}^{-1}$. Animals were tested at 3:00 pm following an 8 h fast. Mice were placed in metabolic cages to collect 24 h urine samples for volume measurement. Urine was stored at -20°C for latter analysis. Urine and serum glucose values were determined spectrophotometrically by the glucose oxydase method [14]. Urine protein concentrations were measured with bovine serum albumin as a standard using a Coomassie dye-binding protein assay [15]. Blood samples were obtained from postorbital vein and centrifuged to get serum. Serum insulin and creatinine were determined by established methods [13,16].

Histological analysis

Animals were killed by cervical dislocation, after which kidneys were quickly removed and placed in normal saline. The middle piece of the left kidney (including the papilla) was fixed in 10% formalin for 24 h at room temperature, dehydrated, and embedded in paraffin for conventional light microscopy. Sections were stained with hemotoxylin-eosin (HE) and periodic-acid Schiff (PAS), respectively. PAS-stained kidney sections were counterstained with hemotoxylin, and were observed by light microscopy (LM) and photographed.

Western blot analyses of renal TGF- β 1

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot to measure renal TGF- β 1 were performed as previously described [17]. Cortex of the right kidney was homogenized and protein was quantified using Bradford method. 60 μ g of protein was separated on SDS-PAGE (5% stacking gel and 15% separation gel). Separated proteins were transferred by electro-elution onto nitrocellulose paper (PROTRAN, Schleicher & Schuell), and incubated with rabbit-anti-mouse specific antibody (sc-146, 1:300 dilution, Santa Cruz Biotechnology) overnight. Membranes were washed with Tris-Tween Buffered Saline (TTBS) and the nitrocellulose sheets were incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate for 1 h. The blots were developed using BCIP/NBT substrate solution, and were quantified by densitometry using BioRad Gel Doc 2000.

Statistical analysis

Data given in the text are mean $\pm$ SEM, with n indicating the number of mice studied. Differences between groups were analyzed by one-way analysis of variance (ANOVA) in combination with the independent-samples t -test when appropriate. $P < 0.05$ was considered significant.

Results

Body weights and serum analysis

Liver glucokinase knockout mice had greater weights than wild-type mice at 6 ($P < 0.05$) and 10 months ($P < 0.01$) but not at 14 months (Fig. 1A). The 10 month old mice showed the greatest difference from wild-type. There was no change in weight between the 10 and 14 month old glucokinase knockout mice, while wild-type mice continued to increase in weight (Fig. 1A). Fasting blood glucose levels were significantly higher in $gck^{w/-}$ mice than in $gck^{w/w}$ mice for all age groups ($P < 0.01$, Fig. 1B). Knockout mice showed serum insulin and creatinine levels that were not significantly different from those of $gck^{w/w}$ mice. The IPGTT displayed a characteristic diabetic curve in $gck^{w/-}$ mice at 6, 10 and 14 months (Table 1). That is, the blood glucose levels at 120 min were significantly higher than those at 0 min ($P < 0.05$), while in wild-type mice 120 min glucose levels were equal to the level at 0 min. As observed with body weight, the 10 month old knockout mice show the greatest excursion in blood glucose levels, with significantly increased 120 min blood glucose levels compared to 14-month $gck^{w/-}$ mice ($P < 0.05$, Table 1).

Urine analysis

In wild-type mice, 24 h urine volume increases with age, with 14 month old mice have significantly greater volumes than either 6 or 10 months (Fig. 2A). A different pattern was observed in glucokinase knockout mice, with 10 month old mice having significantly greater volumes than either 6 or 14 month old mice (Fig. 2A). Comparing glucokinase knockout to wild type mice, at 6 months both had similar volumes, but by 10 months the knockout mice had significantly greater 24 h volume, but at 14 months the wild type mice had a significantly greater 24 h urine volume (Fig. 2A). Urine protein levels were significantly lower ($P < 0.01$) in liver glucokinase knockout mice at 6 months than in wild-type mice (Fig.

2B). By 10 months though, while wild-type mice show a significant ($P<0.01$) reduction in urine protein, glucokinase knockout mice have significantly elevated urine protein levels compared to both 6 month knockout mice and 10 month wild-type mice (Fig. 2B). By 14 months, urine protein levels in knockout mice have reduced significantly, approaching the levels of the wild-type mice (Fig. 2B). Despite the enormous increase in urine volume and the possible signs of proteinuria, these glucokinase knockout diabetic mice showed only small changes in urine glucose levels. There was no significant difference between $gck^{w/w}$ mice and age-matched $gck^{w/-}$ mice. Unexpectedly, the urine glucose of 6-month mice was higher than that of 10- and 14-month mice.

Morphology analysis

Light microscopy of renal tissues from $gck^{w/w}$ showed a normal appearance (Fig. 3A, B&C). A similar morphology was observed in 6-month $gck^{w/-}$ mice (Fig. 3D). In contrast, renal tubular casts were observed in 10- and 14-month $gck^{w/-}$ mice (Fig. 3E&F). There was an age-related increase in PAS-positive materials in mesangial regions. Compared with age-matched $gck^{w/w}$ mice, significant increases in mesangial matrix and thickening of glomerular basement membrane were observed in $gck^{w/-}$ mice at 10 and 14 months of age (Fig. 4E&F). Renal lesions of 10- and 14-month-old $gck^{w/-}$ mice were more severe than that of 6-month-old $gck^{w/-}$ mice, but there was no significant difference between 10- and 14-month-old mice.

Expression of renal TGF- β 1

In both wild-type and glucokinase knockout mice, with increases in age, renal TGF- β 1 levels initially gradually increase, between 6 and 10 months, and then significantly increase between 10 and 14 months (Fig. 5). The increase in TGF- β 1 levels is greater in glucokinase knockout mice, such that, despite similar levels at 6 and 10 months, at 14 months TGF- β 1 levels are significantly greater than those observed in wild-type mice (Fig. 5).

Discussion

In this study, we carried out long-term observation on a liver-specific glucokinase knockout mouse model of MODY2. As previously described, by 6 weeks of age, GCK knockout mice develop hyperglycemia, but their blood glucose levels remain stable [13]. Fasting blood glucose levels are significantly higher in $gck^{w/-}$ mice compared to $gck^{w/w}$ mice. The $gck^{w/-}$ mice have good survival and satisfy the diagnostic criteria for MODY2 [13]. In this study we find that by 14 months of age, blood glucose levels of $gck^{w/-}$ mice show a tendency to decrease, but they were not significantly different from those of 6 and 10 month old $gck^{w/-}$ mice (Fig. 1A). However, compared to age-matched $gck^{w/w}$ mice, blood glucose levels significantly increase at all ages (Fig. 1A). The hyperglycemia associated with GCK mutations is often mild, with fewer than 50% of subjects presenting overt diabetes [3]. Furthermore, basal metabolism in aged mice might be lower than that of 6-month mice, and this may be the cause of the small decrease in blood glucose concentrations.

There were a few mouse models linked to MODY [18,19]. Bali et al used homologous recombination in mouse embryonic stem cells to assess the effects of disrupted GCK

function in both β cells and hepatocytes and generated an animal model for MODY [18]. Our animal model was established using the Cre-loxP gene targeting strategy, in which glucokinase was selectively knocked out in the liver without affecting expression in pancreatic β cells [13], thus our $gck^{w/-}$ mice have normal pancreatic function and serum insulin levels. Impaired glucose tolerance, an early indicator of insulin resistance and diabetes mellitus, signifies the inability of peripheral tissues to perform glucose uptake at rates that are sufficient to prevent excessive post-prandial blood glucose concentrations [20]. Previous research has shown that liver glycogen synthesis is lower in the knockout mice than in age-matched normal mice [21]. In this study, IPGTT displayed an abnormal glucose tolerance curve, which is likely caused by decreased glycogen synthesis due to the reduced liver glucokinase.

Creatinine is a metabolic product of creatine and phosphocreatine, which are both found almost exclusively in muscle [22]. Thus, creatinine production is proportional to muscle mass and varies little from day to day [23]. If extrinsic creatinine intake is stable, then the concentration of blood creatinine depends up on the capacity of glomerular filtration. When the glomerular filtration rate (GFR) decreases to a critical level (typically 1/3 of the normal level), serum creatinine concentration increases dramatically, therefore, it is considered to be an indicator of GFR impairment, although not useful for early diagnosis. Compared to type 1 and type 2 diabetic mice, our GCK knockout mouse has mild hyperglycemia. Because there was no significant difference in creatinine values between $gck^{w/w}$ and $gck^{w/-}$ mice (Fig. 1D), we speculate that its GFR has not decreased to a critical level.

A major deficiency in animal models of DN is the absence of renal failure. Whereas animal models of diabetic kidney disease exhibit albuminuria and some of the characteristic pathologic changes, reports of renal failure resulting from diabetes in mice or rats are lacking [24]. Whether the inability to detect renal failure is simply a consequence of studying mice with established diabetes for an insufficient period of time or that the absence of renal failure reflects an intrinsic resistance to nephropathy of the strains studied thus far remains uncertain.

In diabetes, the appearance of albumin in the urine has long been recognized as a cardinal feature of kidney disease [25]. Albumin cannot diffuse across the glomerulus but glucose can under physiological condition [26]. In this study, it seems that the diffusion of glucose across the glomerular membrane was normal because glucose concentrations did not reach the diagnostic criteria of glucosuria. Currently there is no uniform standard of mouse urine protein concentrations. Carlson found that urine protein can be detected in normal mice, and under fasting conditions, proteinuria of the diabetic mice was lower than proteinuria of controls [27]. Compared with age-matched $gck^{w/w}$ mice, 10-month $gck^{w/-}$ mice have significantly elevated urine volume and protein concentrations (Fig. 2), suggesting that kidney damage exist in 10-month $gck^{w/-}$ mice. Diffuse increased PAS positivity and mesangial expansion in 10- and 14-month mice (Figs. 3 and 4) is evidence for early diabetic nephropathy.

The reasons for reduced levels of proteinuria in 6-month $gck^{w/-}$ mice are unclear (Fig. 2B). Moreover, 14-month $gck^{w/-}$ mice have a lower urine protein concentration than 10-month

$gck^{w/-}$ mice (Fig. 2B). Do they make a recovery? Human DN proceeds through several distinct pathophysiologic stages, including an early stage of glomerular hyperfiltration, followed by the so-called silent phase in which GFR returns to normal [28]. In our study, changes in liver glucokinase should be the main cause. Regulation of GCK expression is complex [29], and we assume that the GCK knockout will affect the expression of other genes. Further studies are being performed to identify these changes.

TGF- β was first discovered as a stimulator of rat kidney cells and its gene family includes multifunctional molecules that have unique properties in cell proliferation, differentiation, migration, development, tissue remodeling and repair [30]. TGF- β 1 is known to be a key cytokine in obesity and insulin resistance and also play pivotal role in the development of diabetic nephropathy with its fibrogenic properties [31]. It is reported that TGF- β signaling events seem to mediate renal matrix expansion in progressive glomerular disease, and the effects of mesangial matrix accumulation may be ameliorated by downregulation of TGF- β target genes in human mesangial cells [32]. However, the role of TGF- β in MODY has not been extensively studied. In our experiments, a significant increase in mesangial matrix and thickening of the glomerular basement membrane was observed in $gck^{w/-}$ mice at 10 and 14 months, so we examined the expression of renal TGF- β 1 and found that TGF- β 1 levels increased (Fig. 5). In $gck^{w/w}$ mice, higher levels of TGF- β 1 in 14-month mice than in 6- and 10-month mice might be an age-related increase (Fig. 5). The significant increase of TGF- β 1 in 14-month $gck^{w/-}$ mice (Fig. 5), is in accord with results from others, suggesting that there are renal changes in our knockout mice, although the renal lesions in the 14-month-old $gck^{w/-}$ mice seemed to be less severe than in 10-month-old $gck^{w/-}$ mice. The lesions, which result from extracellular matrix (ECM) accumulation, were formerly thought to be irreversible [33], however, the quantity of ECM is determined by the rates of production and degradation [34]. By 14 months, TGF- β 1 has significantly increased, which should promote ECM production, but degradation of ECM might be greater than that seen in 10-month-old mice.

Speculations

In the current study, the $gck^{w/-}$ mice have good survival and satisfy the diagnostic criteria for MODY2. That is consistent with Zhang's results. Typical functional and structural renal changes were observed, such as elevated urine protein concentrations and increases in mesangial matrix. We conclude that, our model accurately reflects human diabetic kidney disease and it may provide a valuable model for studying renal disease and its treatment in diabetes.

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Abbreviations

MODY   Maturity-onset diabetes of the young

GCK	glucokinase
DN	Diabetic nephropathy
TGF-β1	transforming growth factor- β 1
IPGTT	intraperitoneal glucose tolerance test
HE	hemotoxylin-eosin
PAS	periodic-acid Schiff
LM	light microscopy
SDS-PAGE	SDS - polyacrylamide gel electrophoresis
TTBS	Tris-Tween Buffered Saline
ANOVA	one-way analysis of variance
GFR	glomerular filtration rate
ECM	extracellular matrix

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Fig 1.

Body weight and blood parameters in $gck^{w/-}$ mice. Body weight (A, $n=6$), blood glucose (B, $n=6$), serum insulin (C, $n=6$), and serum creatinine (D, $n=5-6$) levels were measured in wild type $gck^{w/w}$ (open bars) and $gck^{w/-}$ (solid bars) mice at 6, 10, and 14 months of age.

* $P < 0.05$, ** $P < 0.01$.

Fig 2.

Urinalysis of $gck^{w/-}$ mice. 24 hour urine volume (A, $n=5-6$), urine protein (B, $n=5-6$), and urine glucose (C, $n=4-6$) levels were measured in wild type $gck^{w/w}$ (open bars) and $gck^{w/-}$ (solid bars) mice at 6, 10, and 14 months of age. * $P < 0.05$, ** $P < 0.01$.

Fig 3.

Renal tubular casts in *gck^{w/-}* mice. Renal tissue morphology was examined in wild type *gck^{w/w}* (A, B, C) and *gck^{w/-}* (D, E, F) at 6 (A, D), 10 (B, E), and 14 (C,F) months of age by staining tissue sections with eosin and hemotoxylin. *n*=3 for each analysis.

Fig 4.

Light micrographs of kidney sections in $gck^{w/w}$ and $gck^{w/-}$ mice. All sections were stained with PAS and counterstained with hematoxylin ($n=3$). There was an age-related increase in PAS-positive materials in mesangial regions. Compared with age-matched $gck^{w/w}$ mice, a significant increase in mesangial matrix and thickening of glomerular basement membrane were observed in $gck^{w/-}$ mice at 10 and 14 months of age. A, 6 month $gck^{w/w}$ mice; B, 10 month $gck^{w/w}$ mice; C, 14 month $gck^{w/w}$ mice; D, 6 month $gck^{w/-}$ mice; E, 10 month $gck^{w/-}$ mice; F, 14 month $gck^{w/-}$ mice.

Fig 5.

Expression of renal TGF- β 1 in $gck^{w/-}$ mice. A. Western blot analysis for renal TGF- β 1 from wild type $gck^{w/w}$ (open bars) and $gck^{w/-}$ (solid bars) mice at 6, 10, and 14 months of age. 60 μ g of protein were loaded in each lane and membranes were blotted with anti-TGF- β 1 antibodies (sc-146, Santa Cruz, 1:300). A. Representative blot is shown. B. Quantification of Western blot analysis, $n=3$. The control is the value of TGF- β 1 in 6 months $gck^{w/w}$ mice and has been normalized to β -actin. All values are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

Table 1

Intraperitoneal glucose tolerance test for gck^{w/w} and gck^{w/-} mice

Group	Age (month) (n)	Blood glucose concentration(mmol•L ⁻¹)			
		0 min	30 min	60 min	120 min
gck ^{w/w}	6 (4)	6.85±0.94	20.54±6.31	16.23±2.73	8.44±2.29
	10 (5)	8.70±1.10	20.95±5.30	15.46±1.86	9.24±1.30
	14 (6)	7.36±0.98	26.50±8.64	18.73±6.45	9.00±1.63
gck ^{w/-}	6 (6)	9.74±2.66	29.89±7.42	20.77±3.62	14.06±3.17 [*] , #
	10 (6)	9.47±1.47	29.02±5.70	22.60±5.25	15.80±4.04 ^{**} , ##
	14 (6)	7.54±0.69	31.61±6.70	23.25±6.28	12.37±2.41 [*] , ##

* $P<0.05$ vs age-matched gck^{w/w} mice at 120 min.
** $P<0.01$ vs age-matched gck^{w/w} mice at 120 min.
# $P<0.05$ vs age-matched gck^{w/-} mice at 0 min.
$P<0.01$ vs age-matched gck^{w/-} mice at 0 min.