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The effects of type 1 IGF receptor inhibition in a mouse model of diabetic kidney disease

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Abstract

Objective—We have recently shown increased sensitivity to IGF-I induced signal transduction in kidneys of diabetic mice. Accordingly we investigated the effects of PQ401, a novel diuretic compound that inhibits IGF1R autophosphorylation in type I diabetes.

Methods—Control (C) and Diabetic (D) mice were administered PQ401 (CP, DP) or vehicle (C, D) for 3 weeks.

Results—CP animals showed a decrease in renal phosphorylated (p-)AKT. However, PQ401 had no effect on diabetic state (hyperglycemia, weight loss) or renal disease parameters (hypertrophy, hyperfiltration and albuminuria). Type IV collagen as well as TGF- β mRNA increased in DP and D compared to C. In the CP group renal hypertrophy with fat accumulation in proximal tubuli and increased renal IGF-I, collagen IV and TGF- β mRNA were seen.

Conclusions—IGF1R inhibition by PQ401 exerted no significant effects on diabetic kidney disease parameters, arguing against a role for IGF-I in the pathogenesis of diabetic kidney disease. However, PQ401 affects normal kidneys, inducing renal hypertrophy as well as collagen and fat accumulation, with increased renal IGF-I mRNA, suggestive of a damage-regeneration process. Therefore, this diuretic compound is not beneficial in early diabetic kidney disease. Its potential deleterious effects on kidney tissue need to be further investigated.

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Conflict of Interest Statement

The results presented in this paper have not been published previously in whole or part. We have no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

Keywords

Diabetic nephropathy; Diarylurea; IGF-I; IGF1 receptor; Nephrotoxicity; Non-obese diabetic mice

Introduction

Preclinical research performed over the past 2 decades has suggested growth hormone (GH) and insulin-like growth factors (IGFs) to be involved in the pathogenesis of diabetic kidney disease [1, 2]. In mice with type 1 diabetes mellitus (DM)-serum GH levels are elevated but serum IGF-I and renal IGF-I mRNA levels are decreased, suggesting a GH resistance state [3, 4]. Indeed basal signaling through the GHR/JAK2/STAT5 pathway which regulates IGF-I gene expression is not increased in the kidneys of diabetic mice [5]. Nevertheless, given the presence of GH receptors (GHR) in kidney [6], the increase in circulating GH levels may have a potential role in the development of diabetic nephropathy by activating other signaling pathways [7]. Knock-out (KO) mice with GHR/GH binding protein (GHBP) gene-disruption are protected against DM induced renal changes [8]. Indeed, we and others have previously shown that inhibition of GH, either by the use of a GHR antagonist or a somatostatin analogue has protective renal effects in animals with type I DM [3, 9]. In many animal studies renal IGF-I protein content is increased in type I DM, but renal IGF-I mRNA levels are depressed [10, 11]. Since expression of the main renal IGF binding protein (IGFBP1) is elevated, increased trapping of IGF-I may be the reason for the increase in renal IGF-I protein content. This local increase in renal IGF-I have been suggested to play a role in mediating the development of diabetic kidney disease [12]. In a recent experiment we have shown that following IGF-I administration renal p-AKT, p-rpS6, p-MAPK, and p-GSK [key signaling molecules downstream of type 1 IGF receptor (IGF1R)] levels increased more pronouncedly in diabetic animals suggesting increased sensitivity to IGF-I in DM. In contrast, basal IGF-I receptor (IGF1R) activation was not increased in the DM mice [5]. AKT activation leads to mTOR phosphorylation, eventually leading to increased protein synthesis [13]. The mTOR pathway has been implicated in the pathogenesis of compensatory and diabetic renal growth and treatment with rapamycin (an inhibitor of mTOR) ameliorates diabetic related kidney complications [14].

In breast cancers and certain other malignancies IGF1R plays an important role in promoting the oncogenic process [15–17]. IGF1R autophosphorylation is required for the subsequent activation of IGF1R tyrosine kinase activity. Recently, Gable et al identified a new class of small-molecules called diarylureas that inhibit the IGF1R [18]. One diarylurea compound, PQ401, was found to antagonize IGF1R autophosphorylation and signaling through the AKT pathway and inhibited breast cancer cell growth in culture and in vivo. While these studies suggest that diarylureas acting via inhibition of the IGF1R is potential anticancer agents there is no data about their potential beneficial or adverse effects in other pathologic conditions in which IGF-I may play a role. Given the potential role of IGF-I in the development of diabetic kidney disease, in the present study we examined the effects of PQ401 on the kidneys of diabetic and normal control animals.

Materials and Methods

Animal experimentation

Six weeks old non-obese diabetic (NOD) female mice were used in the experiment (Harlan Laboratories, Indianapolis, USA). Animal breeding complied with the NIH Guide for the Care and Use of Laboratory Animals. The local institutional review committee approved the study protocol. The cumulative incidence of overt DM in these animals is over 70% by 100 days in females. Animals were housed in standard laboratory cages and fed normal mouse chow ad libitum with free access to unlimited amount of tap water. The onset of DM was determined by the appearance and persistence of glycosuria, checked twice a week with chemstrips (Ketostix, Bayer-Ames, UK). When the urine glucose test was positive, tail capillary glucose blood samples were examined with a glucometer (Freestyle, Alameda, CA, USA). DM was diagnosed when blood glucose levels were above the normal values for NOD mice (99% confidence levels of 3.0–9.9 mmol/l) on two consecutive days. The second day of persistent hyperglycemia was considered day 1 of DM. Only mice with elevated serum glucose levels and no ketonuria were included. Non diabetic NOD mice served as controls.

Mice were divided into four groups (7–8 animals per group): control non-diabetic mice (C) and diabetic mice (D) treated with vehicle; control non-diabetic (CP) and diabetic mice (DP) injected with intraperitoneal (IP) PQ401 (kindly provided by Dr. Youngren). PQ401 was dissolved into 50% polysorbate 80/50% ethanol and then diluted with PBS to a final concentration of 4 mg/mL [18]. In a pilot study, treated mice received 50 or 100 mg/kg PQ401 IP thrice a week. The 100 mg/kg dose was lethal to all animals within a period of a week from the injection, but the 50 mg/kg dosage caused no mortality. Control mice received i.p. injections of the polysorbate 80/ethanol/PBS vehicle only. All animals were sacrificed 3 weeks after DM onset. Daily food intake was assessed two days before sacrifice. Urine was collected prior to sacrifice for 24 h, using metabolic cages. Mice were anesthetized with ketamine-xylazine (80 mg/kg of ketamine and 16 mg/kg of xylazine). Prior to sacrifice all groups received an intravenous bolus of analog R-long-IGF-I (200 µg/kg) (GroPep, Adelaide, Australia). Blood was drawn from the retrobulbar plexus 10 min after bolus. The serum was separated and frozen at –80°C. A coronal 2mm slice from the mid portion of the left kidney was separated and fixed in a 4% paraformaldehyde solution for histomorphological assessment. The remaining portions and the right kidney were immediately frozen in –80°C.

Histopathological staining

The midsize portion of kidney tissue (containing the papilla) was fixed in 4% paraformaldehyde and embedded in Technovit. Sections of 2 µm thickness were cut on a rotation microtome and stained with periodic acid-Schiff (PAS) and haematoxylin. In addition, kidney frozen sections were cut to 10–15 µm thickness, then washed in 60% isopropanol and stained in Oil red O and haematoxylin. Photomicrographs were obtained by a light microscope (Zeiss Axioplan MR12, Germany).

Immunoassays

Blood samples were collected and frozen at -80°C until analysis. Serum IGF-I concentrations were measured using an ELISA kit (DSL-10-29200, Diagnostic Systems Lab.). Serum GH concentrations were measured using an ELISA kit (DSL-10-72100, Diagnostic Systems Lab.) by following the manufacturer's instruction. Urine samples were stored at -80° until assayed. Albumin urine excretion was determined by specific mouse albumin ELISA kit (E-90AL, Immunology Consultants Lab.).

Western immunoblot analysis

The IGF1R and p-AKT (Thr308) antibodies were purchased from Santa Cruz Biotechnology (California, USA), antibodies against AKT and p-IGF1R (Tyr1131) were purchased from Cell Signaling Technology (Danvers, MA, USA) and β -actin antibody was purchased from MP Biomedical (Solon, OH, USA).

Kidney lysates were prepared and subjected to western immunoblot analysis as previously described [5]: 100 μg portions of sample protein were electrophoresed on 10% SDS polyacrylamide gel, and electroblotted into nitrocellulose membranes. The blots were incubated overnight at 4°C with the primary antibodies. Subsequently, the blots were incubated with a secondary anti-mouse antibody (β -actin) and anti-rabbit antibody (IGF1R, p-IGF1R, AKT, p-AKT) conjugated to horseradish peroxidase. The band antibody was visualized by the same method as described [5].

RNA extraction and real time-PCR

Total RNA was extracted from kidney as described previously [5] using the PerfectPure RNA Tissue kit (Gentra Systems, Minneapolis, MN). cDNAs were synthesized using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real time PCR (qPCR) assays were performed with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) as described previously [5] using the ABI Prism 7300 Sequence detection System (Applied Biosystems, Foster City, CA.). Primers for quantification of IGF-I, Collagen IV, TGF- β and β -actin (Sigma-Aldrich, Rehovot, Israel) are summarized in Table 1. Each sample was analyzed in triplicate in individual assays. The specificity of the reaction is given by the detection of the melting temperatures (T_{ms}) of the amplification products immediately after the last reaction cycle. The target genes expression value was calculated by the $\Delta\Delta\text{Ct}$ method after normalization with a housekeeping gene (β -actin).

Statistical analysis

One-way analysis of variance was used to evaluate differences between groups for multiple comparisons. The Kruskal–Wallis modification for non-parametric data was used as a first step, and the Mann–Whitney test for differences between the groups was used subsequently. A P -value of <0.05 was considered as significant. Data are depicted as means $\pm$ SEM.

Results

The Diabetic model

NOD female mice became diabetic at age 12–24 weeks. Average glucose levels in D and DP groups were significantly higher than in C and CP groups respectively, but there was no difference in the degree of hyperglycemia between the diabetic groups (Table 2). We did not detect ketonuria in any of the diabetic animals during the study period. Food consumption and urine output were significantly higher in the diabetic animals compared to controls ($p<0.05$) (Table 2). PQ401 treatment had no effect on urine output and food consumption.

GH-IGF-I system

It is known that in type I diabetes models there is a GH resistance state, manifested by elevated serum GH and decreased serum IGF-I. In D and DP groups serum GH levels were significantly higher (341 ± 75 and 389 ± 103 ng/ml) compared to C and CP groups (75 ± 19 and 50 ± 16 ng/ml, $p<0.05$) respectively (Fig 1A). Serum IGF-I levels were significantly lower in D and DP (219 ± 47 and 255 ± 47 ng/ml) compared to C and CP (389 ± 17 and 453 ± 24 ng/ml, $p<0.05$) respectively (Fig 1B). These results show that the type I diabetic model was established and that PQ401 administration had no effect on systemic IGF-I and GH levels.

Kidney p-IGF1R and p-AKT expression

PQ401 has been shown to block IGF1R phosphorylation in a breast cancer cell line. We investigated the effect of PQ401 on IGF1R phosphorylation and the downstream signaling molecule AKT. Kidney p-IGF1R decreased, not statistically significant, in CP compared to C (0.35 ± 0.07 vs 0.7 ± 0.2 AU respectively, $P=0.1$). In DP no decrease in p-IGF1R was seen compared to D (1.4 ± 0.2 vs 1.1 ± 0.1 AU respectively, $p=0.2$) (Fig 2A). Basal p-AKT was increased in D vs C, as we have previously described [5]. Kidney p-AKT decreased by 45% in CP and by 30% in DP (0.6 ± 0.06 , $p<0.05$ and 1.2 ± 0.25 AU) compared to C and D (1.1 ± 0.1 and 1.7 ± 0.9 AU respectively, $p=0.01$) (Fig. 2B). These results suggest that PQ401 has an inhibitory effect on downstream signaling molecules of the IGF1R receptor through AKT but this inhibition was modest in diabetic animals.

Renal hypertrophy

One of the first signs of diabetic kidney disease is kidney hypertrophy and this was also observed in the D group in this experiment. Interestingly, control animals that were treated with PQ401 (CP group) also showed kidney hypertrophy. Kidney weight increased significantly in CP, D and DP (209 ± 6 , 239 ± 9 and 251 ± 14 mg, respectively) compared to C (163 ± 3 mg, $p<0.05$) (Fig 3A). Similarly kidney weight to body weight (KW/BW) (mg/g) increased significantly in CP, D and DP (7.8 ± 0.2 , 9.5 ± 0.3 and 9.5 ± 0.5) compared to C (6.6 ± 0.1 , $p<0.05$) respectively (Fig 3B).

Kidney function and matrix accumulation

To measure kidney function, creatinine clearance (CrCl) and albumin excretion were measured from 24 hr urine collections. CrCl was significantly higher in D (13.8 ± 1.6 $\mu\text{L}/\text{min}/\text{g}$ BW) compared to C and CP mice (7.8 ± 1 and 5.8 ± 1.3 $\mu\text{L}/\text{min}/\text{g}$, $p<0.05$)

respectively (Fig 4A). In the DP group CrCl rate (9.5 ± 2.3 $\mu\text{L}/\text{min}/\text{g}$) was intermediate between D and C and CP (Fig 4A). Albumin excretion was significantly higher in D and DP (1.9 ± 0.6 and 2.3 ± 0.5 $\text{mg}/24$ hr) compared to C and CP (0.15 ± 0.03 and 0.33 ± 0.01 $\mu\text{g}/24$ hr, $p < 0.05$) respectively (Fig 4B). Albumin to creatinine ratio was significantly higher in D compared to C ($364 \pm 47\%$ of C, $p < 0.05$). No significant increase was seen in DP vs CP ($300 \pm 116\%$ and $105 \pm 17\%$ of C respectively, $p = 0.07$) (Fig 4C). PQ401 administration had no effect on the above renal parameters when DP vs D or CP vs C were compared.

Extracellular matrix accumulation (ECM) is an early event in diabetic nephropathy and accompanies kidney hypertrophy. Kidney type collagen IV is a main marker for ECM accumulation in diabetic kidney. Kidney collagen IV mRNA significantly increased in CP, D and DP (2 ± 0.2 , 1.8 ± 0.1 and 2.1 ± 0.3 AU) compared to C (1 ± 0.1 AU, $p < 0.05$) respectively (Fig 5A). Kidney transforming growth factor β (TGF- β) is elevated in diabetes. Kidney TGF- β mRNA was significantly increased in CP, D and DP (1.5 ± 0.1 , 1.6 ± 0.2 and 1.9 ± 0.3 AU) compared to C (1 ± 0.1 , $p < 0.05$) respectively (Fig 5B). Kidney IGF-I mRNA was previously reported to be decreased in diabetes. In our model kidney IGF-I mRNA did not differ between C and D (1 ± 0.2 and 0.8 ± 0.1 AU respectively), but it increased significantly in CP and DP (1.5 ± 0.1 and 2.5 ± 0.6 AU respectively, $p < 0.05$) (Fig 5C). This unanticipated increase in collagen expression induced by PQ401 (possibly controlled by TGF- β) reflects an apparent adverse effect and likely contributes to the renal hypertrophy in the CP group.

Kidney Histology

Periodic acid-Schiff (PAS) staining was performed on kidney sections in order to examine changes in glomerular matrix accumulation. No differences in the thickness of the basement membrane were seen between C and CP compared to D and DP. In the tubular cells of CP animals we noticed foamy changes (Fig 6) that counterstained positive for fat specific Oil red O staining (Fig 7). This phenomenon was not seen in C and D groups and was faint in the DP animals.

Discussion

The local increase in renal IGF-I reported for early type I DM in previous studies [19][20, 21] has been suggested to play a role in mediating the development of diabetic kidney disease. Our previous finding of increased sensitivity to exogenous IGF-I in the diabetic kidney was reflected by the heightened phosphorylated fraction of the signaling molecules AKT, rpS6, MAPK, and GSK. These molecules are associated with suppressed apoptotic signals and increased protein synthesis, including extra cellular matrix (ECM) proteins [5, 22], thus raising the question whether inhibition of renal IGF-I signaling through the IGF1R is beneficial and safe in type I DM.

In order to answer this question and to determine the role of IGF1R in DN we tried to inhibit the IGF1R in type 1 DM mice. We used PQ401, an agent that inhibits IGF1R signaling and cell growth in breast cancer cells in culture and in vivo [18]. The use of an IGF1R antagonist raised the theoretical concern about its possible effects on loss of feedback inhibition on hypophyseal GH secretion [23]. However, PQ401 (at the dose used) had no effect on serum GH and IGF-I levels (Fig 1).

In the current work, treatment with PQ401 showed a trend of reduction in the IGF1R in non-diabetic control mice, statistically not significant, but not in diabetic mice (Fig 2). Interestingly, PQ401 reduced AKT activation in both control and diabetic mice (Fig 2). In spite of the reported properties of PQ401, namely inhibition of IGF1R signaling and cell growth, this compound had no significant therapeutic effect on markers of diabetic kidney disease, namely: renal hypertrophy, hyperfiltration and albuminuria and ECM expression (Figs 4–6). Taken together, these findings may suggest that either this compound (at the chosen dosage and method of administration) lacks a sufficient effect on IGF1R or for the marginal role of IGF-I in the pathogenesis of kidney disease in type I DM [5]. A similar observation has been previously reported for the subtotal nephrectomy model, where a peptide IGF-I antagonist (JB3) was not efficient in suppressing the development of proteinuria, hypertension and renal fibrosis [24].

Apparent nephrotoxic effects were seen in the kidneys of control non-diabetic mice treated with PQ401: there was a significant increase in kidney weight and KW/BW (Fig 3) and increased collagen type IV and TGF- β mRNA levels as well as increased IGF-I mRNA (Fig 5). This response may well represent a damage–repair response. PAS and hematoxylin-eosin staining of kidney sections showed the accumulation of vacuoles in the tubular cells of control treated mice (Fig 6). This phenomenon was seen to a much lesser extent in diabetic treated mice. Oil red O staining of kidney sections revealed that the vacuoles are composed of fat (Fig 7). The origin of this fatty accumulation remains unclear and may reflect nephrotoxicity. Control treated mice (CP group) showed a much higher response to PQ401, manifested by inhibition of IGF1R and AKT activation and fat accumulation, compared to diabetic treated mice. We assume that this relative protection of the DP group may be the result of diabetes and polyuria. Diabetic animals excrete 10 times more urine in 24 hours than control animals (Table 2). This large urine flow could conceivably wash PQ401 from the kidney much faster than in control animals thus reducing its impact on the renal tubules and protecting from any toxic effects.

In type 1 DM mice kidney fat accumulation is associated with increased levels of pro-fibrotic factors such as: TGF- β , VEGF and PAI-1 [25, 26]. In turn these factors cause renal accumulation of ECM proteins including type IV collagen [25, 26]. All this suggest that fat accumulation reflecting a toxic effect of PQ401 administration has a potential to cause kidney damage, even though kidney function did not change following PQ401 treatment. Furthermore, all mice that were treated with a dose of 100 mg/kg (twice the final dosage used in this report) did not survive the treatment. Thus, administration of PQ401 at a higher dose or for a longer period of treatment may eventually cause deleterious effects on kidney function. PQ401 is a new molecule and its effects on other receptors cannot be ruled out. Thus it is not clear which effects might be mediated through alternate cellular targets, or via non-specific mechanisms.

In conclusion, while early reported studies indicate that PQ401 is an effective inhibitor of the IGF1R and cancer cell growth, we have found that it is ineffective in inhibiting diabetic renal growth, ECM accumulation or albuminuria. Although IGF1R phosphorylation was not inhibited in diabetic mice, the overall data argue against a role for IGF-I in the pathogenesis of diabetic kidney disease. Importantly, we have observed that use of this

compound at the tested dose resulted in histologic and molecular signs of nephrotoxicity. While further work is necessary to determine whether this might be a class effect of IGF1R inhibitors, therapeutic development of this or other similarly targeted small molecules must include careful examination of their effects on the kidney.

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Figure 1A-B:

A: Serum GH (ng/ml) (n=5) and **B:** IGF-I levels (ng/ml) (n=7), determined by ELISA. * Statistically significant vs C, $P < 0.05$; # statistically significant vs CP, $P < 0.05$.

Figure 2A-B.

Kidney p-IGF1R and p-AKT determination by Western blotting. **A:** Kidney p-IGF1R/IGF1R ratio, detected in 95–97 KD (n=5). **B:** Kidney p-AKT/AKT, detected in 61 KD (n=5).

* Statistically significant, vs C, $P < 0.05$; # statistically significant, vs CP, $P < 0.05$.

Figure 3A-B.

A: Average kidney weight (mg) (n=7). **B:** Average kidney weight (KW) to body weight (BW) ratio (mg/g) (n=7). * Statistically significant vs C, $P < 0.05$; # statistically significant vs CP, $P < 0.05$.

Figure 4A–C.

A: Creatinine clearance in ($\mu\text{L}/\text{min}/\text{g}$) ($n=7$). **B:** Urine albumin excretion (U-Alb) ($\mu\text{g}/24\text{ hr}$) ($n=5$). **C:** Albumin to Creatinine ratio shown as percentage of control ($n=5$). * Statistically significant vs C, $P < 0.05$; # statistically significant vs CP, $P < 0.05$.

Figure 5A-C.

Real time PCR analysis. Results are presented as fold of control group. **A)** Kidney collagen type IV mRNA (n=6). **B)** Kidney TGF- β mRNA (n=5). **C)** Kidney IGF-I mRNA (n=5).

* Statistically significant vs C, $P < 0.05$; \$ statistically significant vs D, $P < 0.05$.

Figure 6. Kidney histological sections stained with PAS. Green arrow shows tubular cells with foamy accumulation.

Figure 7.
Kidney frozen section stained with Oil red O for fat. Green arrow shows tubular cells with fat accumulation. *G (violet)* - glomerulus

Table 1

Primers used for real time PCR quantification.

Primers	Sequence
TGF- β Sense	AACTATTGCTTCAGCTCCAGAGAG
TGF- β Anti-sense	AGTTGGATGGTAGCCCTTG
IGF-1 Sense	GGCTGCTTTTGTAGGCTTCAGTGG
IGF-1 Anti-sense	GGACCAGAGACCCTTTGCGGGG
Collagen IV Sense	ACTCCACGCAGAGCAGAAGCAAGAA
Collagen IV Anti-	GGTGTACGAATTAGGCAGGTCAAG
β -actin Sense	GGTCTCAAACATGATCTGGG
β -actin Anti-sense	GGGTCAGAAGAATTCCTATG

Table 2

The Diabetic Model. Average blood glucose levels (mg/dl) (n=7), 24 h food consumption (n=8) (g) and 24 h urine output (ml) (n=7).

	C	CP	D	DP
Blood glucose (mg/dl)	103 ± 3	107 ± 7	557 ± 25 *	478 ± 61 #
24 hour Food consumption (g)	6.3 ± 0.5	6.7 ± 1.2	14 ± 1.8 *	14.7 ± 3.1 #
24 hour Urine output (ml)	1.6 ± 0.3	2.3 ± 0.6	24.7 ± 2.7 *	20.3 ± 5.4 #

* Statistically significant, vs C, $P < 0.05$;

statistically significant vs CP, $P < 0.05$.