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ORIGINAL ARTICLE

Increased renal Akt/mTOR and MAPK signaling in type I diabetes in the absence of IGF type 1 receptor activation

Daniel Landau · Renanah Eshet · Ariel Troib · Yotam Gurman · Yu Chen · Ralph Rabkin · Yael Segev

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Abstract Growth hormone (GH) and IGF-I have been implicated in the pathogenesis of type I diabetic (DM) nephropathy. We investigated renal GH receptor (GHR) and IGF-type 1 receptor (IGF1R) signaling in an animal model of type I DM. Kidney tissue was examined for GHR and IGF1R key signaling molecules. GHR levels were unchanged and IGF-I mRNA levels were decreased in the diabetic group (D). Basal and GH stimulated phosphorylated (p-) JAK2 and STAT5 levels were similar in controls (C) and D. The levels of p-IGF1R were similar in the two groups at baseline, while pAkt, pGSK3, p-mTOR, p-rpS6, p-erk1/2 (Mapk), and pSTAT-3 were increased in D. Following IGF-I administration p-Akt, p-rpS6, p-Mapk, and p-GSK levels increased more pronouncedly in D versus C. In conclusion, the lack of JAK2-STAT5 activation and the decrease in kidney IGF-I mRNA levels in D argue against a role for the GH activated JAK2-STAT5 pathway in the pathogenesis of diabetic nephropathy. On the other hand while IGF1R phosphorylation was unchanged, Akt/mTOR and MAPK signaling were hyperactivate in DM, suggesting their involvement. The increase in baseline activated Akt, mTOR, rpS6, and MAPK cannot be explained by activation of the IGF1R, but may be triggered by other growth factors and nutrients.

Keywords Diabetic nephropathy · Growth hormone receptor · JAK2 · Phosphorylation · STAT5

D. Landau (✉)
Pediatric Nephrology, Department of Pediatrics, Soroka Medical Center and Faculty of Health Sciences, Ben Gurion University of the Negev, P.O. Box 151, Beer Sheva 84101, Israel
e-mail: ldaniel@bgu.ac.il

R. Eshet · A. Troib · Y. Gurman · Y. Segev
The Shraga Segal Department Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

Y. Chen · R. Rabkin
Department of Medicine, Stanford University and Research Service, Veterans Administration Health Care System, Palo Alto, CA, USA

Introduction

Diabetic nephropathy (DN) is the most frequent cause of end-stage renal disease in the USA [1]. The growth hormone (GH)-insulin-like growth factor I (IGF-I) system has been implicated in the pathogenesis of DN, based on observations in humans and several rodent models [2]. Serum GH levels are elevated but serum IGF-I and renal IGF-I mRNA levels are decreased in type I diabetes (DM), suggesting a GH resistance state [3]. In many animal experiments renal IGF-I protein content is increased, but renal IGF-I mRNA levels are depressed [4]. Since expression of a main renal IGF binding protein (IGFBP1) is upregulated [5], increased trapping of IGF-I may be the reason for the increase in renal IGF-I content, which may play a role in mediating the development of diabetic kidney disease.

The increase in circulating GH levels may have a potential role in the development of diabetic nephropathy by exerting direct pathological effects through the renal GH receptor (GHR) [6]. Indeed, we and others have previously shown that the inhibition of GH, either by the use of a GHR antagonist [7] or a somatostatin analogue, has protective renal effects in animals with type I DM [8]. In addition, GH administration to diabetic rats had adverse renal effects, such as worsening albuminuria and increased renal IGFBP1 levels, which, as mentioned, may increase the local trapping of IGF-I [9].

Both IGF1R and GHR are expressed in kidney tissue and their signal transduction pathways have been well described [10, 11]. IGF-I and GH may have differing effects on the kidney, exemplified by the different phenotypes of their transgenic mouse models, whereas GH transgenic animals develop significant glomerulosclerosis, IGF-I transgenic animals do not [12].

Given the numerous changes seen in GH and IGF-I systems both in the circulation and in the kidney in animal models of DM, we have investigated the in vivo GH/JAK2/STAT5 and IGF-1R signaling pathways in the kidney of diabetic mice, since hyperactivity of these signaling pathways would implicate these hormones in the pathogenesis of DN.

Results

Induction of diabetes

Intraperitoneal streptozotocin (STZ) induced a DM phenotype in 12-week-old male Balb/c mice, similar to previous descriptions [13], as evidenced by average glucose levels at sacrifice (after 3 weeks of DM) of 364 ± 24 and 412 ± 21 mg/dl, respectively, in the diabetic groups (D and DIGF). For comparison, blood glucose levels in the C and CIGF groups were 110 ± 5 and 117 ± 7 mg/dl, respectively. We did not detect ketonuria in any of the diabetic animals during the study period. Kidney weight to body weight ratio was increased in diabetic mice in comparison to controls (131 ± 4% vs. 100 ± 9% of C; $P < 0.01$).

GH signaling pathway

Renal tissue was examined after 3 weeks of DM. Renal IGF-I mRNA levels were decreased in D animals (70 ± 4% of C value). To understand this decrease in a GH induced key molecule we examined the members of a family of negative regulators of GH and cytokine signaling, SOCS2 and -3. SOCS-3 mRNA levels were elevated in the D kidney (213 ± 35% of C) (Fig. 1) as well as SOCS2 protein (174 ± 15% of C) (Fig. 2). No significant difference in basal GHR, JAK2, and STAT5 protein levels of the untreated DM versus control group was seen (Fig. 2). Stimulation of GHR by intraperitoneal administration of bovine (b-) GH (250 µg/kg) 10 min prior to sacrifice induced a significant elevation in p-JAK2 in diabetic animals and controls. Phospho-STAT5 levels increased following bGH administration in both groups, but did not reach statistical significance in diabetes (Fig. 3). Basal phosphorylated (p-) JAK2 protein levels were reduced in the D group compared to controls (79 ± 6% of control value), while pSTAT5 levels were unchanged (Fig. 3).

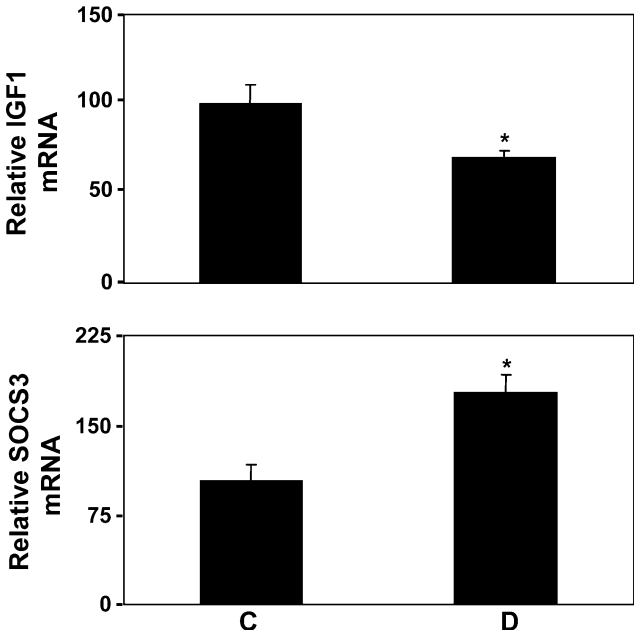


Fig. 1 Kidney IGF-I (*upper panel*) and SOCS3 (*lower panel*) mRNA levels normalized for actin mRNA, in control (C) and diabetic (D) animals measured by real time-PCR after 3 weeks of experimentation. * $P < 0.05$ vs. C

In contrast to pSTAT5, baseline pSTAT3 levels were increased significantly in the diabetic kidney. The p-STAT3/STAT3 ratio in the D kidneys averaged 427 ± 107% of the control value. Administration of bGH did not alter STAT3 phosphorylation in either group. A similar pattern (increase in D vs. C, independent of GH administration) was seen in the IGF-I experiment (Fig. 4). STAT3 is usually activated by GH through JAK2 phosphorylation [14]. As previously mentioned, SOCS-2 protein (Fig. 2) and SOCS-3 mRNA (Fig. 1) levels were also elevated in the D kidney. The absence of basal activation of JAK2/STAT5 in diabetes in our experiment suggests that the increase in pSTAT3 and SOCS2 and 3 expression in diabetes is caused by another member of the cytokine family acting through a different JAK such as JAK1, JAK3, or Tyk [15].

Type 1 IGF receptor signaling

We examined IGF1R signaling molecules in the basal state (comparing C vs. D) and after a single bolus of an IGF-I analogue (long-R IGF1) which does not bind to IGF binding proteins [16]. Kidney IGF-I receptor protein levels were similar in D and C animals (Fig. 5). To measure IGF1R tyrosine phosphorylation we used the only phospho-IGF1R antibody available and this antibody also cross reacts with the phosphorylated insulin receptor. In the basal state the phosphorylated IGF1R/insulin receptor levels did not differ between control and diabetic mice (Fig. 5). In response to a

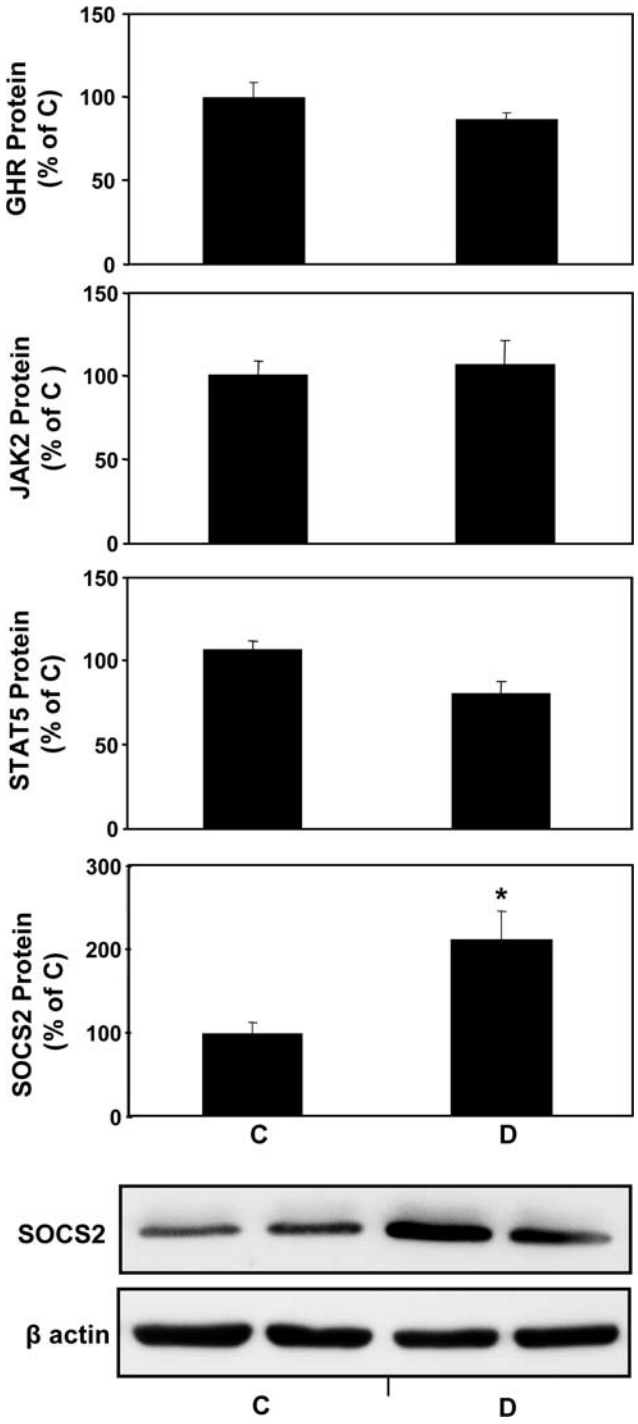


Fig. 2 Kidney GHR, total JAK2, total STAT5, and SOCS-2 proteins in control (C) and diabetic (D) animals measured by western blot analysis. Each graph summarizes five different experiments. * $P < 0.05$ vs. C. *Lower panel* shows a SOCS2 representative gel

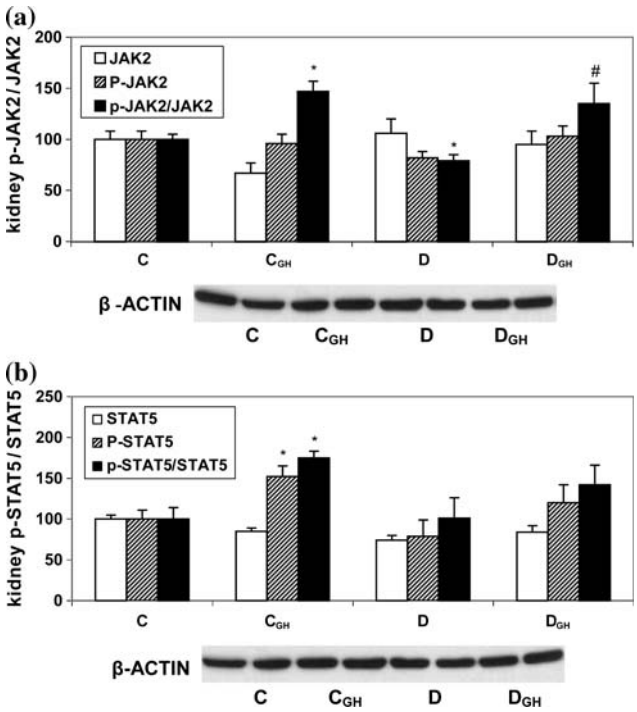


Fig. 3 Relative phosphorylation of JAK2 (**a**) and STAT5 (**b**) in control and diabetic animals 10 min after treatment with saline (C and D groups) or bGH (CGH and DGH groups) prior to sacrifice. Total (*white bar*), phosphorylated (*gray bar*), and phosphorylated-to-total ratio (*black bars*) are depicted for each group. Equal protein loading is shown by a representative gel using β-actin antibody. Each graph summarizes five different experiments. Bars indicate mean ± SEM. * $P < 0.05$ vs. C. # $P < 0.05$ vs. D

single IV dose of long-R-IGF1 (200 μg/kg) 10 min prior to sacrifice the levels of the phosphorylated IGF1R/insulin receptor rose significantly in both groups. While on average the increase in the diabetic group was less than in the controls, the levels were not significantly different (Fig. 5).

Since kidney insulin receptor levels are reported to be downregulated in DM [17] and given the 50–100 higher affinity of IGF-I for the IGF1R than for the insulin receptor [18] and as the dose of long-R-IGF1 administered was submaximal [19], it would seem reasonable to assume that the increase in phosphorylation largely reflects the response of the IGF1R.

The levels of total IRS-1 and its phosphorylated fraction were elevated to the same extent in both diabetic groups (before and after stimulation with long-R-IGF1): total IRS-1 increased to 314 ± 67% and 210 ± 49% of C value in D and DIGF, respectively. Phosphorylated IRS-1 increased to 215 ± 41% and 187 ± 36% of C in D and DIGF, respectively. However, the ratio of phosphorylated/total IRS-1 in these groups was not significantly different from controls (Fig. 6). To further elucidate the mechanism involved in the signaling pathways of IGF1R, the levels of basal and phosphorylated thymoma viral proto-oncogene 1 (p-Akt), p-GSK3 (glycogen synthase kinase 3 beta), p-mTOR (mammalian target of rapamycin), and p-rps6 (ribosomal protein small subunit) were studied by western blot analysis of protein extracts from kidney tissue. As shown in Fig. 7, basal p-AKT to Akt, p-GSK3 to GSK3, p-mTOR to mTOR,

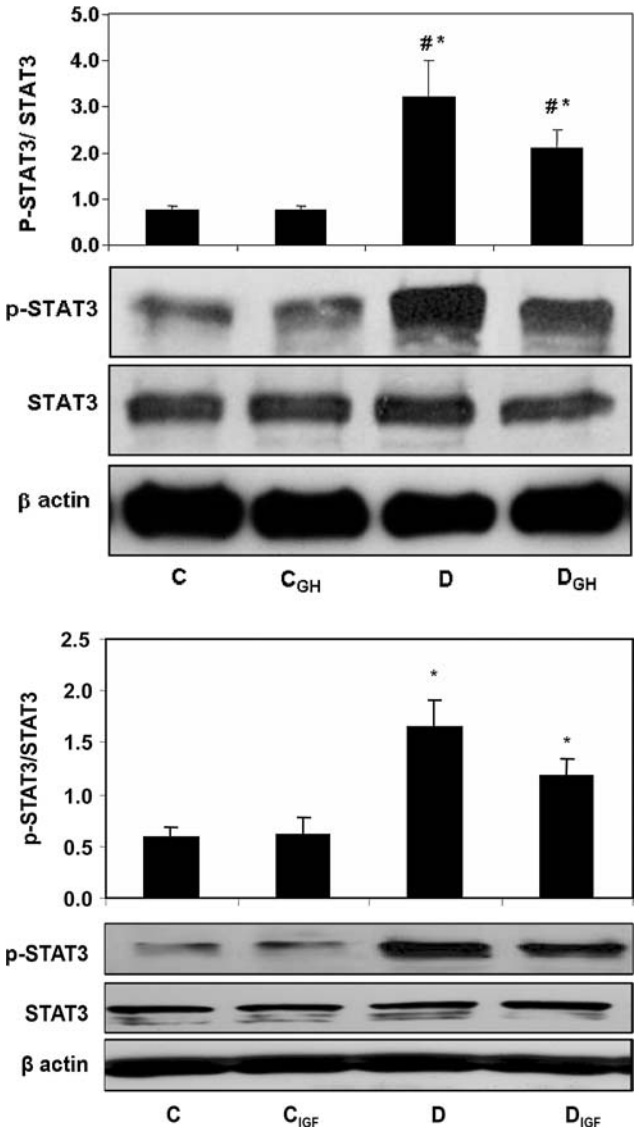


Fig. 4 Relative phosphorylation of STAT3 in control and diabetic animals 10 min after treatment with saline (C and D groups), bGH (CGH and DGH) (*upper panel*), or long-R-IGF-I (CIGF and DIGF groups) (*lower panel*). Phosphoprotein signal was corrected for the total protein level. Each graph summarizes five different experiments. Bars indicate mean ± SEM. A representative gel is depicted under each bar graph. * $P < 0.05$ vs. C. # $P < 0.05$ vs. CGH

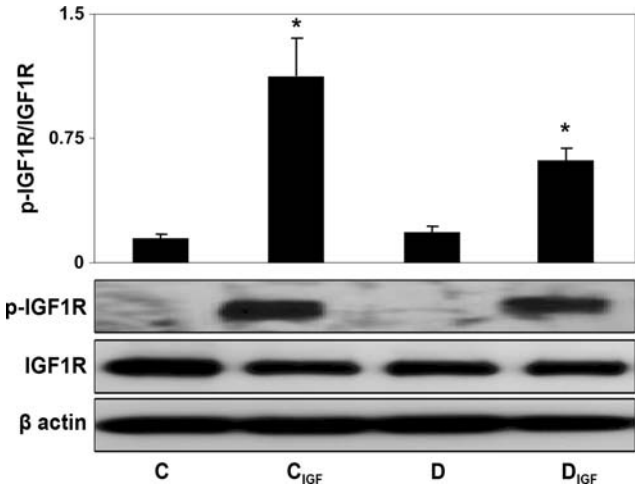


Fig. 5 Relative phosphorylation of IGF1R in control and diabetic animals 10 min after treatment with saline (C and D groups) or long-R-IGF-I (CIGF and DIGF groups). Phosphoprotein signal was corrected for the total protein level. The *upper panel* summarizes five different experiments. Bars indicate mean ± SEM. *Lower panel* shows a representative gel. * $P < 0.05$ vs. C and C

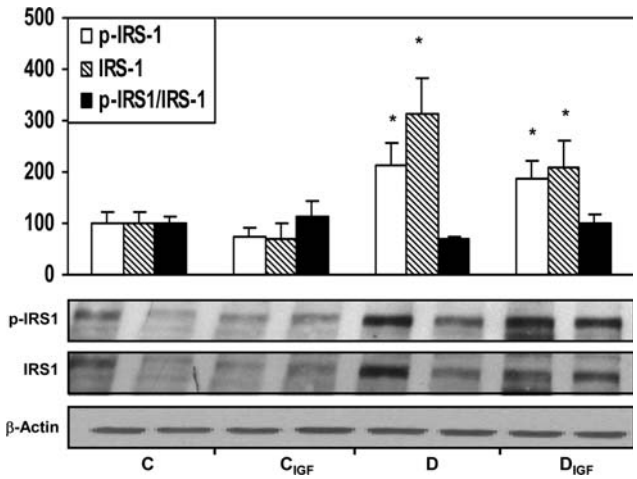


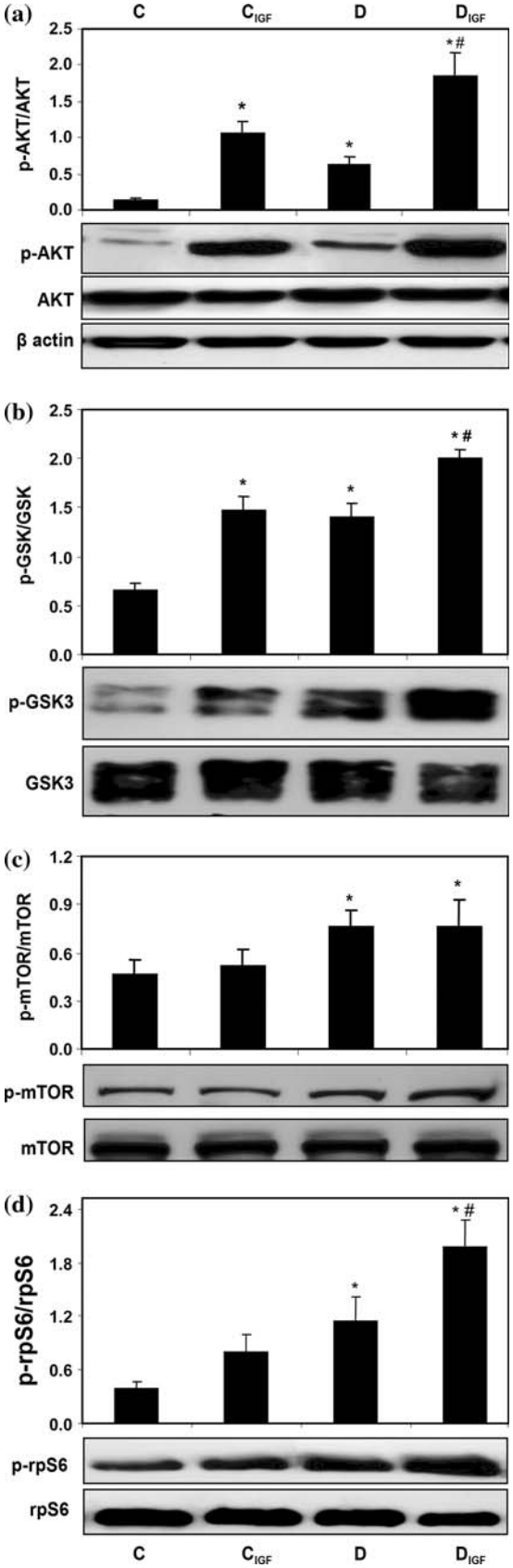
Fig. 6 Relative phosphorylation of IRS1 in control and diabetic animals 10 min after treatment with saline (C and D groups) or long-R-IGF-I (CIGF and DIGF groups). The *lower panel* show representative gels for phosphorylated, total IRS1, and beta-actin. The *upper panel* bar graph summarizes five different experiments. Bars indicate mean ± SEM. * $P < 0.05$ vs. C and CIGF

and p-rps6 to rps6 ratios were increased to 423 ± 70, 224 ± 23, 136 ± 21, and 287 ± 47% of C value in diabetic mice, respectively. Stimulation with long-R-IGF1 caused higher levels of p-AKT, p-GSK3, and p-rps6 in diabetic mice (DIGF group): 1185 ± 214, 294 ± 1, and 613 ± 93% of C, respectively, compared to 683 ± 105%, 229 ± 19%, and 299 ± 59% of C, respectively, in the CIGF groups.

As shown in Fig. 8, the baseline phospho-erk1/2 to erk1/2 ratio was increased in the D kidney (182 ± 27% of C value, $P < 0.05$) and upon stimulation with long-R-IGF1 the levels increased significantly in diabetic animals (221 ± 21% of C value, vs. 124 ± 32% in CIGF).

Discussion

Derangements in the regulation of GH production and action have been implicated in the pathogenesis of distinct glomerular diseases. Human type 1 DM is characterized by an increase in circulating GH and an inverse correlation



◀ **Fig. 7** Relative phosphorylation of **a** AKT, **b** GSK, **c** mTOR and **d** rpS6 in control and diabetic animals 10 min after treatment with saline (C and D groups) or long-R-IGF-I (CIGF and DIGF groups). Each phosphoprotein signal was corrected for its total protein level. Each bar graph summarizes five different experiments. Bars indicate mean ± SEM. Attached to each graph is a representative gel. * $P < 0.05$ vs. C. # $P < 0.05$ vs. D

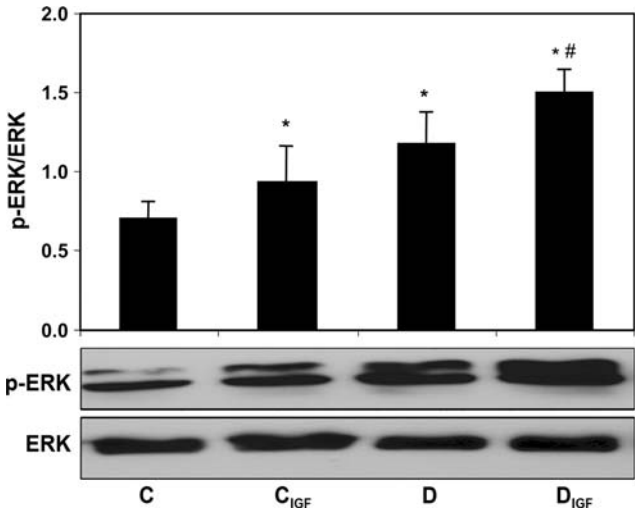


Fig. 8 Relative phosphorylation of erk1/2 (MAPK) in control and diabetic animals 10 min after treatment with saline (C and D groups) or long-R-IGF-I (CIGF and DIGF groups). Phosphoprotein signal was corrected for the total protein level. The *upper panel* summarizes five different experiments. Bars indicate mean ± SEM. *Lower panel* shows a representative gel. * $P < 0.05$ vs. C. # $P < 0.05$ vs. D

between glycemic control and serum GH levels [20, 21]. Several rodent studies have shown that inhibition of GH has preventive effects on the development of diabetic nephropathy. Transgenic mice expressing a GH antagonist are protected from DM-related renal complications [22]. In NOD mice, a model of type 1 DM, circulating GH levels are also increased [2]. GHR levels are depressed in kidney, but to a lesser extent than liver. Kidney IGF-I mRNA levels are reduced in DM but nevertheless extractable renal IGF-I levels are increased, presumably because of increased local trapping by elevated IGFBP1 [3]. In addition, renal cortical GHBP mRNA levels and GHBP immunoreactivity are elevated in STZ-induced DM in rats [4]. Conceivably this increase in the extracellular domain of the GHR could lead to increased trapping of GH in the kidney and together with the increase in circulating GH lead to hyperactivation of the GH signaling pathway. Similarly the increase in local IGF-I could conceivably lead to increased activation of the IGF-I signaling pathway. Together these changes could contribute to the pathological changes in the diabetic kidney. In this study we tested whether signaling through GH and IGF-I activated signaling pathways is actually increased in the diabetic kidney.

In our current study kidney GHR protein levels in diabetic mice were not significantly different from non-diabetic values and signaling through the GHR/JAK2/STAT5 pathway was not increased. Renal IGF-I mRNA levels (the most representative evidence for GH action) were decreased. Basal levels of the GH regulated signaling molecules, JAK2 and STAT5, were also unchanged. Furthermore, phosphorylation of JAK2 and STAT5 increased to a similar extent in diabetic and control animals following the administration of GH. There was a modest but significant reduction in the basal pJAK2 levels in DM and it is possible that this may have been caused by the increase in SOCS2 and SOCS3 expression that occurred (Fig. 2). SOCS2 and SOCS3 are members of a family of negative regulators of GH and cytokine signaling. The origin of SOCS2 and -3 upregulation in the diabetic kidney is unclear but may reflect an increase in the expression and action of other cytokines besides GH [23, 24], and signaling through a JAK other than JAK2, such as JAK1, JAK3, or Tyk [25].

In contrast to pSTAT5, baseline pSTAT3 levels were increased significantly in the diabetic kidney (Fig. 4). JAK2 phosphorylation, which is required for GH induced activation of STAT3, was not increased in DM, suggesting that the increase in pSTAT3 is caused by another member of the cytokine family signaling through a JAK other than JAK2 or possibly through the mTOR pathway [20, 26], which we found to be hyperactive (Fig. 7c). Taken together, the overall picture does not support our operational hypothesis that overactivity of the GHR contributes to the development of kidney disease in murine type I DM.

Since our findings argue against a role of the activated JAK2-STAT5 pathway in the pathogenesis of diabetic nephropathy it is likely that the previously described beneficial effects of GH inhibition on diabetic kidney disease could have been due to inhibition of other pathways activated by GH. For example, Thirone et al. [27] demonstrated that after a sevenfold higher dose of GH than used in the present study, phosphorylation of JAK2 (but not STAT5) and the IRS-1/PI3K/Grb2 arm of signaling increased in kidneys of diabetic rats. In addition, exposure of both murine and human podocytes to GH resulted not just in an increase in the phosphorylation of JAK2 and STAT5 proteins, but also stimulation of focal adhesion kinase, increase in reactive oxygen species, and GH-dependent changes in the actin cytoskeleton [28].

The IGF1R is a ligand-activated tyrosine protein kinase highly homologous to the insulin receptor. Its interaction with IGF-I or -II leads to autophosphorylation of the receptor and downstream proteins, including insulin receptor substrate-1, phosphoinositide 3-kinase, phosphoinositol-3,4,5-trisphosphate, Akt, and several other signaling molecules such as protein kinases C and A. Akt activation leads to mTOR phosphorylation that then activates the 70-kDa ribosomal S6 kinase and ribosomal protein S6, eventually leading to increased protein synthesis [29]. Another important Akt substrate is glycogen-synthase kinase 3 (GSK3), an inhibitor of protein translation suppressed by Akt leading to increased protein synthesis. Increased levels of phosphorylated GSK3 have been associated with suppressed apoptotic signals in mesangial cells exposed to hyperglycemia [30]. The MAPK pathway is another distinct signaling pathway that is activated by IGF-I.

In our study pIRS-1, pAkt, pGSK3, p-mTOR, p-rpS6, p-erk (Mapk), and pSTAT-3 were increased in diabetes, suggesting that overactivity of these signaling molecules may be playing a role in the pathogenesis of DN. Following IGF-I administration, pAkt, p-rpS6, pMAPK, and pGSK levels increased more pronouncedly in D_{IGF} vs. C_{IGF}. Unexpectedly, basal levels of the phosphorylated IGF-I/insulin receptor were unchanged between C and D and upon stimulation with an IGF-I ligand phosphorylation of IGF1R increased similarly in control and diabetic animals. This lack of difference between D and C animals in the degree of basal and IGF-I stimulated phosphorylation of the renal IGF1R does not support our original hypothesis that IGF-I plays a role in the pathogenesis of diabetic nephropathy. However it should be kept in mind that our study examined whole kidney. Thus, we may have missed changes in nephron segments. For example, Tack et al. [31] report a fibrogenic phenotypic change in mesangial cells of diabetic NOD mice, associated with constitutive activation of the IGF-I signaling pathways.

It is noteworthy that the mTOR pathway has been implicated in the pathogenesis of compensatory and diabetic renal growth [32] and treatment with rapamycin (an inhibitor of mTOR) ameliorates diabetic related kidney complications [33, 34]. Since the Akt–mTOR–rpS6 pathway is stimulated by different agents including growth factors and nutrients [35] and not just IGF-I, and as in the diabetic state basal phosphorylation of the IGF1R is not increased while the Akt/mTOR pathway is, we conclude that this pathway is being activated by some factor other than IGF-I. For example, the use of the IGF-I receptor antagonist (JB3) inhibited compensatory kidney growth after subtotal nephrectomy, but had no effect on the development of renal fibrosis, hypertension, proteinuria, or the fall in creatinine clearance [36].

In our study renal phosphorylated Mapk (erk1/2) was increased in DM in the basal state and further increased in DM upon IGF-I stimulation. This increase in basal MAPK activity could be induced by hyperglycemia, which activates stress-activated signaling pathways in diabetic kidney, including nuclear factor-kB, p38 mitogen-activated protein (MAP) kinase, and Jun kinases. Moderate hyperglycemia can activate p38 MAP kinase by a PKC-delta isoform-dependent pathway, and higher glucose levels (>22 mmol/l)

can also activate p38 MAP kinase via a PKC-independent pathway [37]. Treatment with antioxidants and histone deacetylators suppresses this p38 activation and prevents renal hypertrophy in diabetic rats [38]. Interestingly, as with the Akt–mTOR pathway, administration of exogenous IGF-I caused a greater increase in MAPK phosphorylation in the diabetic animals than in the controls. As these two pathways are already hyperactivated in the diabetic kidney, and thus may conceivably be playing a role in the genesis of diabetic kidney disease, caution should be applied when considering the use of IGF-I to control hyperglycemic in DM [39].

In summary, in the diabetic state, the GH responsive renal JAK2/STAT5 signaling pathway was not activated in the basal state and the IGF-I mRNA levels were decreased. Furthermore, the JAK2-STAT5 signaling response to GH was similar to that seen in normal mice. Taken together these findings argue against GH signaling through the JAK/STAT pathway as a cause of diabetic kidney disease, though do not exclude the possibility that GH may be involved in the development of DN through some other signaling pathway. Basal levels of the phosphorylated IGF-I/insulin receptor were similar in C and D and upon stimulation with IGF-I phosphorylation of the IGF-1R increased similarly in C and D animals. This all argues against a role for IGF-I and its receptor in mediating diabetic kidney disease. Of note, baseline levels of activated Akt, mTOR, rps6, and MAPK were increased in the diabetic kidney and rose further after IGF-I administration. Together with the unchanged basal IGF1R phosphorylation this all suggests that the Akt/mTOR signaling pathway and the MAPK pathways are being activated by some factor other than IGF-I and that inhibition of the Akt-mTOR and/or MAPK pathways may turn out to be effective therapeutic targets in diabetic nephropathy.

Methods

Animal experimentation

We purchased 12-week-old male Balb/c mice from Harlan Laboratories (Israel). Animal breeding complied with the National Institutes for Health Guide for the Care and Use of Laboratory Animals. The local institutional review committee approved the study protocol. Animals were housed in standard laboratory cages and had free access to normal mouse chow and unlimited tap water. Diabetes was induced by 6 daily intraperitoneal injections of streptozotocin (STZ) (55 mg/kg). The onset of diabetes mellitus was determined by the appearance and persistence of glycosuria and ketonuria were checked twice a week with chemstrips (Ketostix; Bayer, Newbury, UK). When the urine glucose test was positive, tail capillary glucose blood samples were examined with a glucometer (Elite; Bayer Diagnostics, Puteaux, France). Diabetes mellitus was diagnosed when blood glucose levels were above the normal values for these mice (above 9.9 mmol/l) on two consecutive days. The second day of persistent hyperglycaemia was counted as day 1 of diabetes mellitus.

All animals were killed 3 weeks after the onset of diabetes mellitus. Before sacrifice, mice were anesthetized with ketamine-xylazine (80 mg/kg of ketamine and 16 mg/kg of xylazine). Animals were injected with a single intraperitoneal injection of saline or bovine GH (Monsanto Corp; St. Luis, MO) (250 μg/kg). A second group of animals was injected with intravenous *LONG^{TM}R^{3}IGF-I* (GroPep, Adelaide, Australia) (200 μg/kg) or saline. Both of the injected compounds were given 10 min prior to sacrifice.

RNA extraction and real-time PCR

Total RNA was extracted from kidney using the PerfectPure RNA Tissue kit (Gentra systems, Minneapolis, MN). RNA was treated for DNA contamination using DNase solution (Gentra systems, Minneapolis, MN). RNA concentration was quantified by absorbance at 260 nm. cDNAs were synthesized from 1 μg of total RNA using High-Capacity cDNA reverse transcription kit containing RT random primers, RNase inhibitor, Multiscribe Reverse Transcriptase, dNTP's mix, RT buffer (Applied Biosystems, Foster City, CA, USA). The reaction was incubated at 25°C for 10 min; 37°C for 2 h; 85°C for 5 s. Quantitative real time PCR (qPCR) assays were carried out for IGF-I, SOCS3, and β-actin mRNAs with the following primers: 5′ β actin mouse: GGT,CTC,AAA,CAT, GAT,CTG,GG; 3′ β actin mouse: GGG,TCA,GAA, GAA,T TC,CTA,TG; 5′ SOCS3 mouse: CCC,GCG,GGC,ACC,TT; 3′ SOCS3 mouse: TGA,AGA,AGT,GCA,ACT,CGC,AGT, T; 5′ IGF-I mouse: GGC,TGC,TTT,TGT,AGG,CTT,CAG,T GG; 3′ IGF-I mouse: GGA,CCA,GAG,ACC,CTT,TGC,G GG,G (Sigma-Aldrich, Rechovot, Israel). Primers optimized concentrations were chosen according to primer optimized protocol (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions were performed with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7300 Sequence detection System (Applied Biosystems, Foster City, CA,). Each sample was analyzed in triplicate (final reaction volume 20 μl) in 96-well micro optical plates (Applied Biosystems, Foster City, CA, USA), each sample representing an individual assay. For each sample, 28 ng of cDNA was added to power SYBR green PCR master mix containing Rox (Applied Biosystems, Foster City, CA, USA) and 500 nM primers. The PCR protocol was: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. 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 (IGF-I and SOCS3) expression value was calculated by the ΔΔct method [40] after normalization with housekeeping gene (β-actin).

Western immunoblot analysis

Kidney tissue was homogenized on ice with a polytron (Kinetica, Littau, Switzerland) in lysis buffer (50 mM Tris, pH 7.4, 0.2% Triton X-100) containing 20 mM sodium pyrophosphate, 100 mM NaF, 4 mM EGTA, 4 mM Na3VO4, 2 mM PMSF, 0.25% aprotinin, and 0.02 mg/ml leupeptine. Extracts were centrifuged for 20 mins at 17,000 g at 4°C and the supernatants collected and frozen. Antibodies were purchased for the detection of kidney GHR, SOCS2, IGF-IR, p-AKT (Thr308), erk1/2 (Santa Cruz Biotechnology, CA, USA); p-IRS1, JAK2 (Upstate, Lake placid, NY, USA), p-JAK2 (pYpY1007/1008) (Biosourse, Camarillo, CA, USA); IRS1, AKT, mTOR, GSK 3β, STAT3, rp-S6, p-STAT5 (Tyr694), p-IGF-IR (Tyr1131), p-mTOR (Ser2448), p-GSK 3α/β (Ser21/9), p-STAT3 (Tyr705), p-rp-S6 (Ser235/236) (Cell signaling Technology Inc. Denvers, MA), p-erk1/2 (Thr183 and Tyr185) (Sigma-Aldrich, St.Luis, MO), and β-actin (MP Biomedical, Solon, OH, USA). Homogenates were mixed with 5× sample buffer and boiled for 5 min. Then, 150 μg portions of sample protein were loaded in each gel lane and subjected to 7.5–10% SDS polyacrylamide gel, and electroblotted into nitrocellulose membranes. Blots were blocked for 1 h in TBST (0.05% Twin-20) buffer (10 mM tris, pH 7.4, 138 mM NaCl) containing 5% non-fat dehydrated milk, followed by overnight incubation with polyclonal antibody diluted in TBST (0.05% Twin-20) containing 5% dry milk. The phosphorylated antibodies were diluted in TBST (0.05% Twin-20) containing 5% BSA (MP Biomedical, Solon, OH, USA). After washing 3 times for 15 min in TBST (0.05% Twin-20), the blots were incubated with a secondary anti-goat antibody (GHR), anti-mouse antibody (STAT3, p-erk1/2, β-actin), anti-rabbit antibody (JAK2, p-JAK2, SOCS2, IGF-IR, p-IGF-IR, AKT, p-AKT, M MTOR, p-MTOR, GSK3, p-GSK3, erk1/2, p-STAT3, rpS6, p-rpS6) conjugated to horseradish peroxidase for 1 h at room temperature and then washed again 3 times. The band antibody was visualized by enhanced chemiluminescence (ECL; Amersham, Life Sciences Inc.) and exposed to Kodak-BioMax film (Eastman Kodak, Rochester NY, USA). Protein expression was quantitated densitometrically using Fluorchem software (Alpha-Innotech, California, USA).

Statistical analysis

Four to six animals were used per group. Data are depicted as mean ± s.e.m. Comparisons between more than two normally distributed groups were made by one-way analysis of variance followed by pair wise multiple comparisons with the Holms t-test [41]. A P value <0.05 was considered significant. In the text the results are expressed as a percentage of the average control value.

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