Modulation of Growth Hormone Signal Transduction in Kidneys of Streptozotocin-Induced Diabetic Animals

Effect of a Growth Hormone Receptor Antagonist

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Growth hormone (GH) and IGFs have a long distinguished history in diabetes, with possible participation in the development of renal complications. The implicated effect of GH in diabetic end-stage organ damage may be mediated by growth hormone receptor (GHR) or postreceptor events in GH signal transduction. The present study investigates the effects of diabetes induced by streptozotocin (STZ) on renal GH signaling. Our results demonstrate that JAK2, insulin receptor substrate (IRS)-1, Shc, ERKs, and Akt are widely distributed in the kidney, and after GH treatment, there is a significant increase in phosphorylation of these proteins in STZ-induced diabetic rats compared with controls. Moreover, the GH-induced association of IRS-1/PI3K, IRS-1/Grb2, and Shc/Grb2 are increased in diabetic rats as well. Immunohistochemical studies show that GH-induced p-Akt and p-ERK activation is apparently more pronounced in the kidneys of diabetic rats. Administration of G120K-PEG, a GH antagonist, in diabetic mice shows inhibitory effects on diabetic renal enlargement and reverses the alterations in GH signal transduction observed in diabetic animals. The present study demonstrates a role for GH signaling in the pathogenesis of early diabetic renal changes and suggests that specific GHR blockade may present a new concept in the treatment of diabetic kidney disease. Diabetes 51:2270–2281, 2002

Diabetic nephropathy is one of the most common causes of end-stage renal failure in the Western world (1,2). Renal and glomerular hypertrophies are observed in the early phase of human and experimental diabetes (3,4), and the search for significant pathogenic mechanisms in diabetic kidney disease has focused on these early events. Growth hormone (GH) and IGFs have a long and distinguished history in diabetes, with possible participation in the development of renal complications (5,6). Accordingly, in early experimental diabetes, an initial transient increase in kidney IGF-1 is observed, followed by renal and glomerular hypertrophy (7,8). Also, diabetic dwarf rats are characterized by diminished renal and glomerular hypertrophy compared with diabetic control animals with intact pituitary (9,10), and administration of the long-acting somatostatin analog octreotide to streptozotocin (STZ)-induced diabetic animals inhibits renal and glomerular hypertrophy (7). In addition, specific changes occur in the renal GH binding protein mRNA, IGF-1 receptor mRNA, and IGF binding protein mRNA expression in long-term diabetes (5–10).

The growth hormone receptor (GHR) itself is not a tyrosine kinase (11), but after receptor binding, JAK2, a member of the Janus family of tyrosine kinases, is activated after its association with a dimerized GHR in response to hormone binding (12). As a consequence of the kinase activation, GH stimulates the tyrosyl phosphorylation of some substrates, such as insulin receptor substrate (IRS)-1 (13–16), IRS-2 (16,17), and Shc proteins (16,18). Tyrosyl phosphorylation of IRSs in response to GH provides binding sites for specific proteins containing SH2 domains, including the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (13–16). Downstream from PI3K, the pleckstrin homology domain–containing serine-threonine kinase Akt is activated, and its phosphorylation appears to be the primary mechanism by which enzymatic activity is regulated (19). Similarly, GH promotes the binding of growth factor receptor bound 2 (Grb2) to IRS-1 (16,20) and Shc proteins (16,18). It was also demonstrated that GH stimulates the mitogen-activated protein (MAP) kinase/ERK (21) and that one of the pathways that leads GHR to MAP kinase involves Grb2, son of Sevenless, Ras, Raf, and MAP kinase-ERK kinase (22,23).

In the present study, GH-induced phosphorylation of JAK2, IRS-1, Shc, ERKs, and Akt and association of IRS-1/PI3K, IRS-1/Grb2, and Shc/Grb2 were examined in kidneys of STZ-induced diabetic rats 4 days and 8 weeks after STZ administration. In addition, the effect of G120K-PEG (a genetically engineered analog of GH produced by a mutation in the third α-helix that blocks GH action, preventing GHR dimerization) on renal enlargement and GH signal transduction in kidneys of diabetic mice was evaluated.
TABLE 1

Body weight, blood glucose, and kidney weight in male Wistar rats

<table>
<thead>
<tr>
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<th>4 days</th>
<th>Diabetes</th>
<th>Control</th>
<th>8 weeks</th>
<th>Diabetes</th>
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<tr>
<td>Blood glucose (mg/dL)</td>
<td>112 ± 4</td>
<td>323 ± 18*</td>
<td>115 ± 5</td>
<td>400 ± 19†</td>
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<tr>
<td>Body weight</td>
<td>176 ± 5</td>
<td>153 ± 4*</td>
<td>276 ± 7</td>
<td>121 ± 11†</td>
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<tr>
<td>Kidney weight</td>
<td>750 ± 3</td>
<td>870 ± 4*</td>
<td>819 ± 30</td>
<td>901 ± 29†</td>
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<tr>
<td>Kidney weight/body weight</td>
<td>4.22 ± 0.09</td>
<td>5.68 ± 0.04*</td>
<td>2.96 ± 0.08</td>
<td>7.44 ± 0.4†</td>
<td></td>
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Data are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. control 8 weeks.

RESEARCH DESIGN AND METHODS

Materials. Male Wistar rats and male Swiss mice were obtained from the UNICAMP Central Breeding Center (Campinas, Sao Paulo, Brazil). Monoclonal antiphosphotyrosine antibody (αP/Cleone 4G10) was from Upstate Biotechnology (Lake Placid, NY). Anti-JAK2 (αJAK2/HR-758), anti-IRS-1 (αIRS-1/C-20), anti-Shc (αShc/C-20), anti-PDK (αPDKα585-Z8), anti-Grb2 (αGrb2/C-23), anti-P-ERK (αERK/Ty204), anti-ERK2 (αERK2/C-14), and anti-Akt1 (αAkt1/C-20) antibodies were from Santa Cruz Technology (Santa Cruz, CA). Anti-α-Actin (serine 473) antibody was from New England Biolabs (Beverly, MA). Routine reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Human biosynthetic GH (Norditropin) was purchased from Novo Nordisk (Bagsvaerd, Denmark). 125I-Protein A was from Amersham (Amersham, U.K.). G120K-PEG was from Sensus Drug Development Corporation (Austin, TX). Materials for immunostaining were from Vector Laboratories (Burlingame, CA).

STZ treatment. Diabetes was induced in 6-week-old male Wistar rats by a single intravenous injection of STZ (Sigma; 60 mg/kg in citric buffer, pH 4.5) and in 6-week-old male Swiss mice by a single intraperitoneal injection of STZ (Sigma; 100 mg/kg in citric buffer, pH 4.5). Age-matched normal animals received an equivalent volume of citric buffer, pH 4.5, and were served as control groups. Rats were used for the experiments 4 days or 8 weeks after receiving STZ injection, and mice were used 4 days after STZ treatment. The body weight of the animals was recorded, and diabetes was confirmed by a blood glucose level >250 mg/dl. Plasma glucose levels were determined by the glucose oxidase method using blood samples obtained from the animal tail before the experiments were performed. All experiments involving animals were approved by the University of Campinas ethical committee.

G120K-PEG treatment. One diabetic group of mice was treated with subcutaneous injections of a pegylated GHR antagonist (G120K-PEG) started 24 h after STZ injection, and mice were used 4 days after STZ injection. Blood glucose (mg/dL) 112 153 323 819 819 121 400 121 276 115 400 19 Body weight 176 153 4 870 2 76 276 121 11 1 4 68 11 19 Kidney weight 750 870 4 5 68 0.04 5.68 6 2.96 7.44 7.44 0.4 Kidney weight/body weight 4.22 5.68 0.04 2.96 0.08 7.44 0.4

Data are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. control 8 weeks.

RESULTS

Rat characteristics. Data on body weight, plasma glucose levels, and kidney weight are given in Table 1. At 4 days and 8 weeks after STZ injection, body weight of STZ-treated diabetic rats had significantly decreased when compared with the control group, and plasma glucose concentration was markedly more elevated in STZ-treated diabetic rats than in controls. Kidney weight and the kidney weight expressed relative to body weight increased in both 4-day and 8-week diabetic animals when compared with those in control groups.

Effect of STZ-induced diabetes on GH-stimulated JAK2 phosphorylation level in the kidneys of rats. To determine the level of JAK2 phosphorylation, solubilized proteins from rat kidney stimulated by GH were immunoprecipitated with αJAK2 and immunoblotted with antiphosphorylated tyrosine antibody. There was a significant increase in the GH-stimulated phosphorylation of JAK2 in the diabetic rats 4 days after STZ injection when compared with the controls (diabetic rats, 193 ± 12%; vs. controls, 100 ± 25% P < 0.01) (Fig. 1A). When the same blots were reprobed with αJAK2 antibody, an increase was observed in the level of this protein in the diabetic rats 4 days after STZ injection (diabetic rats, 154 ± 10%; vs. controls, 100 ± 14%; P < 0.01) (Fig. 1B). GH-induced JAK2 tyrosyl phosphorylation was also increased in diabetic rats after 8 weeks of STZ treatment (diabetic rats at 8 weeks, 181 ± 00%; vs. controls at 8 weeks, 100 ± 18% P < 0.01) (Fig. 1A), and JAK2 protein expression was also increased in this group of animals (diabetic rats at 8 weeks, 162 ± 08%; vs. controls at 8 weeks, 100 ± 16% P < 0.01) (Fig. 1B).
Effect of STZ-induced diabetes on GH-stimulated IRS-1 phosphorylation and on IRS-1/PI3K and IRS-1/Grb2 association levels in the kidneys of rats. There was a significant increase in the GH-stimulated tyrosyl phosphorylation of IRS-1 in STZ-induced diabetic rats when compared with the control animals after 4 days (diabetic rats, 189 ± 10%, vs. controls, 100 ± 21%; P < 0.005) (Fig. 1C) and after 8 weeks of STZ treatment (diabetic rats at 8 weeks, 178 ± 10%, vs. controls at 8 weeks, 100 ± 20%; P < 0.005).

Tyrosyl phosphorylation of IRS-1 in response to GH provides binding sites for Grb2, a protein linked to mitogenic pathways (16,20). Similarly, there was an increase in the amount of Grb2 that coprecipitated with IRS-1 in STZ-induced diabetic rats when compared with the controls after 4 days (diabetic rats, 145 ± 9%, vs. controls, 100 ± 15%; P < 0.05) and after 8 weeks of diabetes (diabetic rats at 8 weeks, 155 ± 10%, vs. controls at 8 weeks, 100 ± 14%; P < 0.05) (Fig. 1E). Using the specific anti-peptide antibody against IRS-1 (Fig. 1F), the expression of this protein was found to be unchanged in the kidneys of rats treated with STZ.

Effect of STZ-induced diabetes on GH-stimulated Shc phosphorylation and on Shc/Grb2 association levels in the kidneys of rats. To better define the extent of Shc phosphorylation, we performed a Western blot analysis of

FIG. 1. GH-stimulated JAK2 and IRS-1 tyrosine phosphorylation in kidney of control (C) and STZ-induced diabetic (D) rats after 4 days and 8 weeks (C8 and D8) of diabetes onset. Kidney extracts from rats injected with GH (+) were prepared as described in RESEARCH DESIGN AND METHODS. Tissue extracts were immunoprecipitated with αJAK2 (2 µg/ml) and immunoblotted with αPY (1 µg/ml) (A) and αJAK2 (1 µg/ml) (B). Tissue extracts were also immunoprecipitated with αIRS-1 (2 µg/ml) and immunoblotted with αPY (1 µg/ml) (C). The same blot was incubated with αPI3K (1 µg/ml) (D), αGrb2 (1 µg/ml) (E), and αIRS-1 (1 µg/ml) (F). These results are represented as the means ± SE of scanning densitometry of seven experiments. *P < 0.05 vs. C; †P < 0.05 vs. C8. IB, immunoblotting; IP, immunoprecipitation.
tyrosyl-phosphorylated proteins in anti-Shc immunoprecipitates after stimulation with GH in both groups of animals (Fig. 2A). After stimulation with GH, increased tyrosyl phosphorylation of a protein migrating at an Mr (molecular weight) of 52,000 (appropriate for Shc) was observed in both groups of animals, but comparison of the bands stimulated by GH revealed that the extent of phosphorylation of Shc was increased in STZ-induced diabetic rats compared with controls after 4 days (diabetic rats, 29%, vs. controls, 16%; P < 0.05) and after 8 weeks of STZ treatment (diabetic rats at 8 weeks, 22%, vs. controls at 8 weeks, 18%; P < 0.05). Shc protein expression was not significantly different in all groups of rats (Fig. 2C).

To investigate the effect of diabetes induced by STZ treatment on Shc/Grb2 association in kidneys of rats, Shc and associated proteins were immunoprecipitated with anti-Shc and Western blotted with anti-Grb2 (Fig. 2B). A protein recognized by αGrb2 in Western blots and migrating with the appropriate size for Grb2 (Mr 23,000) was precipitated by αShc. We observed that after GH stimulation, the amount of Grb2 associated with Shc was increased in STZ-induced diabetic rats compared with controls at 4 days (diabetic rats, 10%, vs. controls, 11%; P < 0.05) and 8 weeks after diabetes was induced (diabetic rats at 8 weeks, 28%, vs. controls at 8 weeks, 18%; P < 0.05). Shc protein expression was not significantly different in all groups of rats (Fig. 2C).

Effect of STZ-induced diabetes on GH-stimulated ERKs and Akt phosphorylation levels in the kidneys of rats. To estimate the rate of GH-induced ERK phosphorylation in the kidney of STZ-induced diabetic rats, we performed experiments with whole tissue extracts from all animals (Fig. 2D). By immunoblotting the nitrocellulose membranes with anti-p-ERK antibody, we observed that GH-stimulated ERK phosphorylation was increased in the kidney of diabetic rats compared with control animals at 4

FIG. 2. GH-stimulated Shc, ERK, and Akt phosphorylation in kidney of control (C) and STZ-induced diabetic (D) rats after 4 days and 8 weeks (C8 and D8) of diabetes onset. Kidney extracts from rats injected with GH (+) were prepared as described in RESEARCH DESIGN AND METHODS. Tissue extracts were immunoprecipitated with αShc (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (A), αGrb2 (1 μg/ml) (B), and αShc (1 μg/ml) (C). Whole tissue extracts containing the same amount of protein were separated with SDS-PAGE, and after transfer to nitrocellulose, blots were probed with α-ERK (1 μg/ml) (D), αERK2 (E), α-Akt (1 μg/ml) (F), and αAkt (G). These results are represented as the means ± SE of scanning densitometry of seven experiments. *P < 0.05 vs. C; †P < 0.05 vs. C8. IB, immunoblotting; IP, immunoprecipitation.
days (diabetic rats, 155 ± 12%; vs. controls, 100 ± 13%; \( P < 0.05 \)) and 8 weeks after diabetes was induced by STZ treatment (diabetic rats at 8 weeks, 151 ± 11%; vs. controls at 8 weeks, 100 ± 14%; \( P < 0.05 \)). There was no change in ERK protein expression in the kidney of diabetic rats compared with controls (Fig. 2E).

We also established the level of Akt serine phosphorylation in the kidney of STZ-induced diabetic rats by immunoblotting the nitrocellulose membranes with samples obtained from the whole tissue extracts, which contained anti-\( \text{p-Akt} \) antibody (Fig. 2F). Comparison of the bands stimulated by GH revealed that the extent of serine phosphorylation of Akt was increased in STZ-induced diabetic rats compared with controls after both 4 days (diabetic rats, 167 ± 20%; vs. controls, 100 ± 11%; \( P < 0.05 \)) and 8 weeks (diabetic rats at 8 weeks, 171 ± 22%, vs. controls at 8 weeks, 100 ± 15%; \( P < 0.05 \)) of STZ treatment. When the same membranes were probed with \( \alpha \text{Akt} \), the expression of this protein was the same in all groups of animals studied (Fig. 2G).

**Tissue distribution of JAK2, Shc, and IRS-1 in kidney of control and STZ-treated rats.** In kidneys of both controls (4 days and 8 weeks) and STZ (4 days and 8 weeks) rats, JAK2 was presented with a wide distribution in cells of the glomeruli, proximal, and distal tubuli and collecting tubuli. As shown in Fig. 3 (upper right inset), JAK2 staining was present in most cells of kidneys in controls, with an apparent increase in intensity of staining in STZ-induced diabetic animals at 4 days and 8 weeks. In glomeruli of 4-day STZ rats, strongly anti-JAK2-stained mesangial cells were observed with high frequency. Similarly, histological characterization of Shc distribution in kidney revealed a wide distribution, with positive staining in cells of the glomeruli, and all types of tubuli and collecting tubuli. As shown in Fig. 3 (lower left inset), of major importance was the observation of an increase in magnitude Shc staining of mesangial cells in glomeruli of 4-day STZ rats compared with glomeruli from 4-day control rats. Finally, the expression of IRS-1 followed a pattern similar to that observed for JAK2 (Fig. 3, lower right inset). Thus, there was a wide distribution of IRS-1 staining in glomeruli, and all types of tubuli, with more pronounced staining localized to collecting tubuli. An apparent increase in expression was observed in 4-day and 8-week STZ-induced rats compared with their respective controls. Nonetheless, despite an apparent increase in magnitude Shc and IRS-1 staining in kidney cells of STZ rats compared with controls, no increase in Shc or IRS-1 protein expression was detected by immunoblotting.

**Histological characterization of GH-induced phosphorylation of Akt and ERK in kidney.** Attempting to gain further information on the main sites and characteristics of the response to GH in kidneys of control and diabetic rats, immunohistochemical staining using \( \text{p-Akt} \) and \( \text{p-ERK} \) antibodies was performed (Fig. 4). In samples collected after treatment with saline, a basal, low-intensity, wide staining was detected in control and STZ (4 days and 8 weeks) rats for either \( \text{p-Akt} \) or \( \text{p-ERK} \). After GH treatment, an increase in the staining signal was detected in samples collected from animals belonging to all groups studied. \( \text{p-Akt} \) staining was more sharply detected than \( \text{p-ERK} \) in all samples analyzed. For \( \text{p-Akt} \), the more important difference in staining was detected between 8 weeks STZ-saline and 8 weeks STZ-GH (Fig. 4, lower left inset). The presence of \( \text{p-Akt} \) was detected in most cells of the kidney, with apparent highest staining evidenced in collecting tubuli and some cells of the glomerular mesangium. \( \text{p-ERK} \) staining was more difficult to be detected, however; as shown in Fig. 4 (upper right inset), the highest stimulus response was detected between STZ-saline/4 days and STZ-GH/4 days.

**Morphometric analysis of glomeruli.** Glomeruli of STZ-treated rats presented, at regular hematoxylin-eosin staining, some histological differences compared with their counterparts in the kidneys of control rats. An apparent increase in glomerulus size was evident in kidney sections form 8-week STZ animals; moreover, an apparent thickening of the basement membrane and visualization of loop-like structures occupying large extensions of most glomeruli was evident and probably due to mesangial expansion (not shown). To evaluate the mean volume of glomeruli in kidneys of animals from each group, a morphometric analysis was performed. No difference was detected in mean glomerulus volume between 4-day control (2.60 ± 0.14 \( \mu m^3 \times 10^6 \)), 8-week control (2.54 ± 0.15 \( \mu m^3 \times 10^6 \)), and 4-day STZ (2.62 ± 0.17 \( \mu m^3 \times 10^6 \)) rats. However, the comparison of glomerular mean volume of rats belonging to the 8-week STZ group (3.04 ± 0.18 \( \mu m^3 \times 10^6 \)) with any of the other groups revealed a significant difference, with an increase of ~17% in mean volume (\( P < 0.001 \)).

**Mice characteristics.** G120K-PEG has specificity for mice. For this reason, we performed the subsequent experiments using mice. The severity of diabetes was similar in both the G120K-PEG–treated and untreated mice because similar plasma glucose and body weights were seen in the two groups (Table 2). Kidney weight was significantly increased in the diabetic mice 4 days after STZ injection. G120K-PEG treatment, however, prevented the increase in kidney weight (Table 2).

**Effect of G120K-PEG on GH-stimulated JAK2 phosphorylation level in the kidneys of diabetic mice.** To investigate the effects of G120K-PEG on GH-induced JAK2 tyrosyl phosphorylation in kidneys of diabetic mice, kidney samples from the three groups of mice were immunoprecipitated with anti-JAK2 antibody and immunoblotted with antiphosphotyrosine antibody. The results showed that GH-stimulated JAK2 tyrosyl phosphorylation was increased in the diabetic mice that did not receive G120K-PEG compared with control animals (diabetic mice, 188 ± 26%, vs. controls, 100 ± 8% \( P < 0.05 \)). When diabetic mice were treated with G120K-PEG, GH-induced JAK2 tyrosyl phosphorylation was similar to that of control mice (D+Tx, 108 ± 8%, vs. controls, 100 ± 8%; \( P = 0.5 \)) (Fig. 5A).

STZ treatment produced an increase in the JAK2 protein level in mice kidney, as demonstrated when the same blots were probed with \( \alpha \text{JAK2} \) (diabetic mice, 155 ± 22%, vs. controls, 100 ± 12% \( P < 0.05 \)) (Fig. 5B). This increase was prevented by G120K-PEG treatment because the JAK2 protein level was quite the same in STZ diabetic animals treated with the antagonist compared with that of control mice (D+Tx, 104 ± 12%, vs. controls, 100 ± 10%; \( P = 0.5 \)) (Fig. 5B).
Effect of G120K-PEG on GH-stimulated IRS-1 phosphorylation and on IRS-1/P13K and IRS-1/Grb2 association levels in the kidneys of diabetic mice. We also tested the effect of G120K-PEG on IRS-1 phosphorylation in kidney of diabetic mice, performing a Western blot analysis of the tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates after stimulation with GH in the three groups of animals. In accordance with the behavior of JAK2, GH-stimulated IRS-1 tyrosyl phosphorylation was increased in the diabetic mice compared with control animals (diabetic mice, 198 ± 15%, vs. controls, 100 ± 6%; P < 0.001) and returned to values similar to those of the control group, when diabetic mice were also treated with G120K-PEG (D+Tx, 112 ± 10%, vs. controls, 100 ± 6%; P = 0.33) (Fig. 5C). The blots that had been previously immunoprecipitated
with antibody against IRS-1 were subsequently incubated with antibodies against the 85-kDa subunit of PI3K (Fig. 5D). The comparison of the bands stimulated by GH revealed that the amount of PI3K associated with IRS-1 was increased in STZ-induced diabetic mice when compared with the controls (diabetic mice, 155 ± 10%; vs. controls, 100 ± 11%; P < 0.01) and was indistinguishable from controls in G120K-PEG–treated animals (D+Tx, 95 ± 6%, vs. controls, 100 ± 11%; P = 0.7).

To determine the extent of IRS-1/Grb2 association in kidney extracts from the three groups of animals, we performed experiments by stripping and reprobing the same membrane above with anti-Grb2 antibody. The level of IRS-1/Grb2 association was increased in the
TABLE 2
Body weight, blood glucose, and kidney weight in male Swiss mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + G120K-PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>135 ± 4</td>
<td>423 ± 11*</td>
<td>396 ± 16*</td>
</tr>
<tr>
<td>Body weight</td>
<td>30.5 ± 0.7</td>
<td>26.5 ± 0.6*</td>
<td>26 ± 1.2*</td>
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<td>Kidney weight</td>
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<td>190 ± 8*</td>
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<td>Kidney weight/body weight</td>
<td>6.5 ± 0.3</td>
<td>8.7 ± 0.3*</td>
<td>7.3 ± 0.1†</td>
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Data are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. diabetes.

diabetic mice compared with control animals (diabetic mice, 148 ± 14%, vs. controls, 100 ± 8%; P < 0.05) and did not differ from controls in G120K-PEG–treated mice (D+Tx, 99 ± 13%, vs. controls, 100 ± 8%; P = 0.94) (Fig. 5E). Figure 5F shows that there was no change in IRS-1 protein expression in the three groups studied.

Effect of G120K-PEG on GH-stimulated Shc phosphorylation and on Shc/Grb2 association levels in the kidneys of diabetic mice. To examine the effect of G120K-PEG on GH-induced Shc tyrosyl phosphorylation in kidneys of diabetic animals, kidney samples from the three groups of mice were immunoprecipitated with anti-Shc antibody and immunoblotted with antiphosphotyrosine antibody. GH-induced Shc tyrosine phosphorylation in diabetic mice was increased compared with that of control animals.

FIG. 5. GH-stimulated JAK2 and IRS-1 tyrosine phosphorylation in kidney of control (C), STZ-induced diabetic (D), and G120K-PEG–treated STZ-induced diabetic (D+Tx) mice. Kidney extracts from mice injected with GH (+) were prepared as described in RESEARCH DESIGN AND METHODS. Tissue extracts were immunoprecipitated with αJAK2 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (A) and αJAK2 (1 μg/ml) (B). Tissue extracts were immunoprecipitated with αIRS-1 (2 μg/ml) and immunoblotted with αPY (1 μg/ml) (C). The same blot was incubated with αPI3K (1 μg/ml) (D), αGrb2 (1 μg/ml) (E), and αIRS-1 (1 μg/ml) (F). These results are represented as the means ± SE of scanning densitometry of five experiments. *P < 0.05 vs. C; †P < 0.05 vs. D. IB, immunoblotting; IP, immunoprecipitation.
animals (diabetic mice, 192 ± 31% vs. controls, 100 ± 13%; P < 0.05). This increase in Shc tyrosine phosphorylation was almost eliminated in the G120K-PEG–treated diabetic mice (D+Tx, 111 ± 10%, vs. controls, 100 ± 13%; P = 0.52) (Fig. 6A).

Because Shc can associate with Grb2 after GH stimulation, blots with samples that had been previously immunoprecipitated with anti-Shc were reprobed with anti-Grb2. The GH-induced association between Shc/Grb2 increased in the diabetic mice compared with control animals (diabetic mice, 150 ± 10%, vs. controls, 100 ± 10%; P < 0.01) and decreased to the control levels when animals were treated with G120K-PEG (D+Tx, 92 ± 12%, vs. controls, 100 ± 10%; P = 0.62) (Fig. 6B). As with IRS-1, there was no change in Shc protein expression in the kidney after GH stimulation between the groups (Fig. 6C).

**Effect of G120K-PEG on GH-stimulated ERKs and Akt phosphorylation levels in the kidneys of diabetic mice.** We also tested the effect of G120K-PEG on GH-induced ERK phosphorylation in the kidneys of STZ-induced diabetic rats by immunoblotting the nitrocellulose membranes with anti-p-ERK antibody. The results showed that ERK phosphorylation was increased in the kidneys of diabetic mice compared with control animals (diabetic mice, 154 ± 15%, vs. controls, 100 ± 11%; P < 0.05), and G120K-PEG reversed the effect of diabetes and decreased the phosphorylation of ERKs to control levels (D+Tx, 103 ± 13%, vs. controls, 100 ± 11%; P = 0.86) (Fig. 6D).

When the same blot was reprobed with anti-ERK2 antibody, no differences were observed in the level of this protein in all groups (Fig. 6E).

Similar results were observed for Akt phosphorylation because GH-induced Akt serine phosphorylation in the kidney of untreated diabetic mice was increased compared with that of control animals (diabetic mice, 174 ± 22%, vs. controls, 100 ± 13%; P < 0.05). This increase in
GH-induced Akt serine phosphorylation was almost eliminated in the G120K-PEG-treated diabetic mice (D+Tx, 109 ± 15%, vs. controls, 100 ± 13%; P = 0.66) (Fig. 6F). STZ and G120K-PEG treatment produced no significant changes in the Akt protein expression in mice kidneys, as demonstrated when the same blots were probed with α-Akt (Fig. 6G).

DISCUSSION

In the present study, it was demonstrated that GH-induced tyrosyl phosphorylation of JAK2, IRS-1, and Shc was increased in kidneys of 4-day and 8-week STZ-induced diabetic rats and mice, suggesting that these increases in the first steps of GH signal transduction may play a role in diabetic nephropathy. Initial renal growth in experimental diabetes is characterized by glomerular hypertrophy, followed by tubular hypertrophy and hyperplasia. Immunohistochemistry studies demonstrated that in the kidney, the proteins JAK2, IRS-1, Shc, ERK, andAkt are widely distributed and apparently suffer regulation in animals with diabetes. The increase in JAK2 protein expression may be an important mechanism that allows for an increase in GH signal transduction in kidneys of diabetic animals. Our report is in accordance with other studies that support a role for GH in diabetic kidney disease, given that GH-deficient rats with diabetes are relatively protected from typical renal effects of diabetes seen in GH-sufficient rats (9,26), whereas transgenic mice expressing excess GH develop glomerular hypertrophy, albuminuria, and glomerulosclerosis—a sequence of events similar to the evolution of diabetic nephropathy (27). Similarly, transgenic mice expressing excess IGF-1 binding protein have elevated GH levels and developed mesangial hypertrophy and glomerulosclerosis, despite a decrease in plasma IGF-1 levels (28). These studies are consistent with a GH-dependent process by which diabetic nephropathy is mediated, and our results provide data on GH signal transduction that reinforces previous information.

Recent studies have shown that increased diacylglycerol (DAG) levels initiated by hyperglycemia and activation of protein kinase C (PKC) are associated with many vascular abnormalities in retinal, renal, and cardiovascular tissues (29–31). GH has been shown to elicit rapid transient increases in DAG in multiple cell types (32,33). In rat adipocytes, GH-induced DAG production is blocked by a tyrosine kinase inhibitor, genistein (33), which also inhibits JAK2 (34). This is consistent with GH-dependent PKC activation lying downstream of JAK2 in these cells. Our results, showing that in kidney of diabetic animals there is an increase in GH-induced JAK2 tyrosine phosphorylation levels, may suggest another pathway that may lead to DAG-PKC activation in these animals.

In this article, we also demonstrate that there is an increase in GH-induced IRS-1 tyrosine phosphorylation and IRS-1/Pi3K association in kidneys of diabetic rats. Pi3K is the best studied signaling molecule activated by IRS-1. It plays an important role in the regulation of a broad array of biological responses, including membrane ruffling, mitogenesis, and differentiation (35). Different approaches have demonstrated that Akt/protein kinase B (PKB), a serine-threonine kinase with a pleckstrin homology domain, is functionally located downstream of Pi3K (19,36) and has been implicated in the mediation of some of the insulin and GH actions (37–39). Through Western blot, an increase in GH-induced phosphorylation levels of Akt in diabetic animals was observed, which was further supported by histological studies showing a more pronounced staining of p-Akt in kidney of 8-week diabetic animals, with the apparent highest staining evidenced in collecting tubuli and some cells of the glomerular mesangium. Because Akt/PKB phosphorylation is closely related to Akt/PKB activity, we suggest that Akt/PKB activity induced by GH is probably increased in diabetic rat kidneys.

In addition to PI3K, tyrosine phosphorylated motifs in the IRS protein bind to the SH2 domains in several small adapter proteins, including Grb2 (16,20). Flanking its SH2 domain, Grb2 contains SH3 domains that associate constitutively with mSos, a guanine nucleotide exchange protein that stimulates GDP/GTP exchange on p21ras (22,23). The recruitment by growth receptors of Grb2/mSos to membranes containing p21ras is one of the mechanisms used to activate the MAP kinase cascade. During GH stimulation, Grb2 engages IRS-1 and Shc, which results in activation of the MAP kinase isoforms ERK1 and ERK2 (21). Because a signal driven by Shc/Grb2 and IRS-1/Grb2 leads to Ras and MAP kinase activation and positively modulates multiple cellular events linked to development, growth, and mitogenesis, these results suggest that increased activity of the IRS-1 and Shc branches of the GH signaling pathway can be an addition to the mitogenic effect of GH playing a role in the diabetic nephropathy. Activation of the MAP kinase pathway is thought to lead to selective changes in gene transcription and protein synthesis and is therefore likely to be crucial for cellular responses to GH (21,23). Early diabetic nephropathy is characterized by excessive growth and fractional expansion of the glomerular mesangium (4). MAP kinase pathway activation has been implicated in the development of proliferative glomerular disease (40–43), resulting in increased gene expression and enhanced synthesis of extracellular matrix proteins, including type I and IV collagens, fibronectin, and laminin (44). Although p-ERK staining was more difficult to detect, the highest stimulus-response difference was detected between STZ-saline/4 days and STZ-GH/4 days. The localization of some GH-induced signaling events to scattered mesangial cells of the glomerulus, which was more pronounced at day 4 of STZ treatment, may play a role in diabetes-linked glomerular growth. In the present experiments, the morphometric analysis revealed a significant increase in glomerular volume at 8 weeks after STZ treatment. The increased volume was accompanied by architectural changes that are characteristic of the diabetic kidney. We propose that early GH signaling in mesangial cell participates actively in diabetes-induced glomerular growth, which is anatomically evident after a longer period of time.

By recognizing the potential role that GH may play in various pathophysiological conditions, including diabetic nephropathy, a series of highly specific antagonists of GH action has been recently developed for potential therapeutic use (45). Thus, previous studies have described renoprotective effects of GH antagonists in long-term diabetic transgenic mice that express GH antagonists (26,46,47). Our results demonstrate normalization of diabetic renal
enlargement in diabetic mice treated with G120K-PEG and an inhibitory effect on GH-induced tyrosyl phosphorylation of the proteins involved in GH signal transduction, showing that the increase in JAK2, IRS-1, Shc, ERK, and Akt phosphorylation and that the increase in IRS-1/Pt3K, IRS-1/Grb2, and Shc/Grb2 associations in 4-day STZ-induced diabetic animals can be prevented by this GHR antagonist. Accordingly, two recent studies showed protection against diabetes-associated kidney damage: in transgenic mice expressing a disruption in the GH receptor and in diabetic mice with a polyethylene-glycol GHR antagonist (49). Therefore, the results provide evidence for an important role of GH in the development of diabetes-induced end-organ damage, implying that modulation of GH effects may have beneficial therapeutic implications in diabetic nephropathy. Similar results have been previously published with long-acting somatostatin analogs, which have a well-known inhibitory effect on the early increase in renal IGF-1 concentration and renal size in diabetes (7). In GH antagonist-treated diabetic mice, in parallel with the prevention of renal enlargement and glomerular hypertrophy, renal IGF-1 accumulation was also abolished (50), suggesting a similar final effect of octreotide and GH antagonist.

In summary, the results of this study demonstrate that GH-induced phosphorylation of JAK2, IRS-1, Shc, ERK, and Akt and IRS-1/Pt3K, IRS-1/Grb2, and Shc/Grb2 associations are increased in kidneys of 4-day and 8-week STZ-induced diabetic rats. These data suggest that diabetes is characterized by an increase in the GH signaling that might contribute to the development of diabetic kidney disease. Also, administration of G120K-PEG in diabetic mice has inhibitory effects on diabetic renal enlargement and in the increased activity of the GH signaling pathway observed in diabetic animals. The present study demonstrates alterations in the early steps of GH signal transduction in kidneys of diabetic rats and mice and suggests that specific GH blockade may prevent these alterations and may represent a new concept in the treatment of diabetic kidney disease.

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