Inhibitory Effect of a Growth Hormone Receptor Antagonist (G120K-PEG) on Renal Enlargement, Glomerular Hypertrophy, and Urinary Albumin Excretion in Experimental Diabetes in Mice

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Growth hormone (GH) and IGFs have a long and distinguished history in diabetes, with possible participation in the development of renal complications. To investigate the effect of a newly developed GH receptor (GHR) antagonist (G120K-PEG) on renal/glomerular hypertrophy and urinary albumin excretion (UAE), streptozotocin-induced diabetic and nondiabetic mice were injected with G120K-PEG every 2nd day for 28 days. Placebo-treated diabetic and nondiabetic animals were used as reference groups. Placebo-treated diabetic animals were characterized by growth retardation, hyperphagia, hyperglycemia, increased serum GH levels, reduced serum IGF-I, IGF-binding protein (IGFBP)-3, and liver IGF-I levels, increased kidney IGF-I, renal/glomerular hypertrophy, and increased UAE when compared with nondiabetic animals. No differences were seen between the two diabetic groups with respect to body weight, food intake, blood glucose, serum GH, IGF-I, and IGFBP-3 levels or hepatic IGF-I levels. Kidney IGF-I, kidney weight, and glomerular volume were normalized, while the rise in UAE was partially attenuated in the G120K-PEG–treated diabetic animals. No effect of G120K-PEG treatment on any of the parameters mentioned above was seen in nondiabetic animals. In conclusion, administration of a GHR antagonist in diabetic mice has renal effects without affecting metabolic control and circulating levels of GH, IGF-I, or IGFBP-3, thus indicating that the effect of G120K-PEG may be mediated through a direct inhibitory effect on renal IGF-I through the renal GHR. The present study suggests that specific GHR blockade may present a new concept in the treatment of diabetic kidney disease. ***Diabetes*** **48:377-382, 1999**

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Received for publication 5 March 1998 and accepted in revised form 19 October 1998.

W.F.B., J.J.K., and J.A.S. hold stock in Sensus. J.A.S. serves on the board of directors for Sensus.

bGH, bovine growth hormone; GH, growth hormone; GHBP, growth hormone–binding protein; GHR, growth hormone receptor; IGFBP, IGF-binding protein; rGH, rat growth hormone; RIA, radioimmunoassay; STZ, streptozotocin; UAE, urinary albumin excretion.

Of all new cases of end-stage renal failure, ~30% are attributable to diabetic nephropathy, making it one of the most common causes of end-stage renal failure in the Western world (1–4). The early changes in diabetic kidney disease are characterized by an increase in kidney size, glomerular volume, and kidney function and later on by the development of mesangial proliferation, accumulation of glomerular extracellular matrix, increased urinary albumin excretion (UAE), and glomerular sclerosis. Overt diabetic nephropathy is clinically characterized by proteinuria, hypertension, and a progressive renal insufficiency (1–4). The search for significant pathogenic mechanisms in diabetic kidney disease has focused on the early events, at a time point when the pathophysiological changes mentioned above take place. Growth hormone (GH) and IGFs have a long history in diabetes (5–10), with conceivable effects on the development of diabetic renal complications (5–10). Accordingly, diabetic dwarf rats are characterized by diminished renal and glomerular hypertrophy and an increase in UAE when compared with diabetic control animals with intact pituitary (11,12). In line with this, administration of a long-acting somatostatin analog (octreotide) to streptozotocin (STZ)-induced diabetic animals with normal pituitary inhibits renal and glomerular hypertrophy and UAE (13–18). In addition, the initial increase in renal size and function in experimental diabetes is preceded by a rise in renal IGF-I, IGF-binding proteins (IGFBPs), and IGF-II/mannose-6-phosphate receptor concentrations (9–25). Finally, specific changes occur in the renal GH-binding protein (GHBP) mRNA, IGF-I receptor mRNA, and IGFBP mRNA expression in long-term diabetes (9–25). In the present study, the effect of a novel GH receptor (GHR) antagonist (G120K-PEG) on renal enlargement, glomerular hypertrophy, and UAE was examined in STZ-induced diabetic mice.

RESEARCH DESIGN AND METHODS

Animals and protocol. Four groups of adult female Balb/C(a) mice (Bomholtgaard, Denmark) with initial body weights of 15–19 g were studied. Mice were housed 7–8 per cage in a room with a 12:12 h (0700–1900) artificial light cycle, a temperature of 21 ± 2°C and humidity of 55 ± 2%. Animals had free access to standard food (Altromin #1324, Lage, Germany) and tap water throughout the experiment. Animals were randomized into four groups; two of these groups were made diabetic by a single intravenous injection of STZ (Upjohn, Kalamazoo, MI) in a dose of 300 mg/kg body wt. Blood glucose was measured at day 0 (day of STZ injection) and days 1, 7, 14, 21, and 28 in tail-vein blood by Haemoglucotest 1-44 and Reflolux II reflectance meter (Boehringer Mannheim, Mannheim, Germany), and urine was tested for glucose and ketone bodies by Neostix-4 (Ames, Stoke Poges, Slough, U.K.). Only animals with blood glucose levels >16 mmol/l, with

urine glucose concentrations >111 mmol/l, and without ketonuria were included in the study. One nondiabetic group and one diabetic group were treated with subcutaneous injections of a pegylated GHR antagonist (G120K-PEG) (Sensus, Austin, TX) (26–28). Treatment was started 24 h after STZ injection and was given in a dose of 2 mg/kg body wt every 2nd day to maintain high diurnal levels of the GHR antagonist. Untreated diabetic and nondiabetic control mice were injected with an equivalent volume of vehicle (0.154 mol/l NaCl). Every week during the study, the following parameters were recorded: body weight, blood glucose, fodder consumption, and urinalysis for glucose and ketone bodies.

At 4 days before death, animals were placed in individual metabolic cages for determination of 24-h UAE. By the end of the study period (day 28), animals were anesthetized with sodium barbital (10 mg/kg i.p.), and blood was drawn exactly 5 min later from the retro-orbital venous plexus for determination of serum GH, G120K-PEG, IGF-I, and IGFBP-3. Serum was stored at –80°C until measurements were performed. Furthermore, the right kidneys were rapidly removed and carefully cleaned and weighed. A 2-mm thick slice cut horizontally from the middle of the right kidney (including the papilla) was fixed in 4% paraformaldehyde for morphological measurements. Finally, the left kidneys and liver tissue were snap-frozen in liquid nitrogen for later determination of IGF-I.

Immunoassays. Serum GH was measured by radioimmunoassay (RIA) using a specific polyclonal rabbit rat(r) GH antibody and rGH as standard. Semilog linearity of mouse serum and rGH (in the standard) was found at multiple dilutions, indicating antigen similarity between mouse GH and rGH. The ingredients, including ^{125}I-labeled rGH, were obtained from Amersham (Amersham International, Bucks, U.K.). Serum levels of G120K-PEG were measured by an in-house human GH RIA as previously described (29). Serum IGF-I was measured after extraction with acid-ethanol. The mixture was incubated for 2 h at room temperature, centrifuged, and 25 µl of the supernatant was diluted 1:200 before analysis. Kidney and liver extraction was performed as previously described (11,13,15). Briefly, 80–100 mg of tissue was homogenized on ice in 1 mol/l acetic acid (5 ml/g tissue) with an Ultra Turrax TD 25 and further disrupted using a Potter Elvehjelm homogenizer (both from Janke-Kunkel, Staufen, Germany). With this procedure, all IGFBPs are removed from kidney tissue, and an additional extraction procedure (with ethanol/HCl) was performed in liver homogenates to fully remove all IGFBPs. After lyophilization, the samples were redissolved in phosphate buffer (pH 8.0) and kept at –80°C until the IGF-I assay was performed in diluted extracts. Serum and tissue (kidney and liver) IGF-I levels were measured by RIA using a polyclonal rabbit antibody (Nichols Institute Diagnostics, San Capistrano, CA) and recombinant human IGF-I as standard (Amersham International). Tissue IGF-I concentrations were corrected for the contribution of entrapped serum IGF-I (14). Mono-iodinated IGF-I (^{125}I-labeled [Tyr^{31}]IGF-I) was obtained from Novo Nordisk (Bagsvaerd, Denmark). Intra- and interassay coefficients of variation were <5% and <10% for both assays.

Western ligand blotting for determination of serum IGFBP-3. SDS-PAGE and Western ligand blotting were performed according to the method of Hossenlopp et al. (30) as previously described (24). Serum (2 µl) was subjected to SDS-PAGE (10% polyacrylamide) under nonreducing conditions. The electrophoresed proteins were transferred by electroelution onto nitrocellulose paper (Schleicher & Schuell, Munich, Germany), and membranes were incubated overnight at 4°C with ~500,000 cpm ^{125}I-labeled IGF-I (specific activity 2,000 Ci/mmol) in 10 ml/10 mmol/l Tris-HCl buffer containing 1% bovine serum albumin and 0.1% Tween (pH 7.4). Membranes were washed with Tris-buffered saline, and after drying overnight, the nitrocellulose sheets were autoradiographed with Kodak X-AR film and exposed to Du Pont-NEN (Boston, MA) enhancing screens at –80°C for 3–7 days. Specificity of the IGFBP bands was ensured by competitive coincubation with unlabeled IGF-I purchased from Bachem (Bubendorf, Switzerland). On Western ligand blotting (with ^{125}I-IGF-I as ligand), IGFBP-3 appears as a 38–42 kDa doublet band corresponding to the intact acid-stable IGF-binding subunit of IGFBP-3. Western ligand blots were quantified by densitometry using a Shimadzu CS-9001 PC dual-wavelength flying spot scanner.

Estimation of glomerular volume. A 2-mm thick horizontally cut slice from the middle of the right kidney (containing the papilla) was fixed in 4% paraformaldehyde and embedded in Technovit. Then, 2-µm thick sections were cut on a rotation microtome and stained with *p*-aminosalicylic acid and hematoxylin. Mean glomerular tuft volume (V_G) was determined from the mean glomerular cross-sectional area (A_G) by light microscopy as previously described (31,32). Profile areas were traced using a computer-assisted morphometric unit (Image Tool; University of Texas Health Science Center, San Antonio, TX). A_G was determined as the average area of a total of 40–80 glomeruli (tuft omitting the proximal tubular tissue within the Bowmann capsule), and V_G was calculated as

$$V_G = \beta/k \times (A_G)^{3/2}$$

where $\beta = 1.38$, which is the shape coefficient for spheres (the idealized shape of glomeruli), and $k = 1.1$, which is a size distribution coefficient (31,32).

UAE. Urinary albumin concentration in 24-h urine collections was determined by RIA as previously described (14), using rat albumin antibody and standard. Semilog linearity of mouse urine and rat albumin (in the standard) was found at multiple dilutions, indicating antigen similarity between mouse albumin and rat albumin. Urine samples were stored at –20°C until the assay was performed. Rabbit anti-rat albumin antibody, RARa/Alb, was purchased from Nordic Pharmaceuticals and Diagnostics (Tilburg, Netherlands). For standard and iodination, a globulin-free rat albumin was obtained from Sigma (St. Louis, MO).

Statistics. Analysis of variance for repeated measurements was used in evaluation of differences in combination with Student's t test for unpaired comparisons. A P value <0.05 was regarded as significant. Data are given as means ± SE.

RESULTS

Body weight, metabolic parameters, and food consumption. Body weight gains in placebo-diabetic mice were reduced compared with those in nondiabetic mice ($P < 0.01$), and no effect of G120K-PEG treatment was seen in any of the groups (Fig. 1*A*). Blood glucose levels were markedly elevated in the two diabetic groups 24 h after STZ injection (24-h data not shown), with mean values around 20 mmol/l throughout the study period (Fig. 1*B*). G120K-PEG administration in nondiabetic and diabetic animals did not affect blood glucose levels when compared with respective control

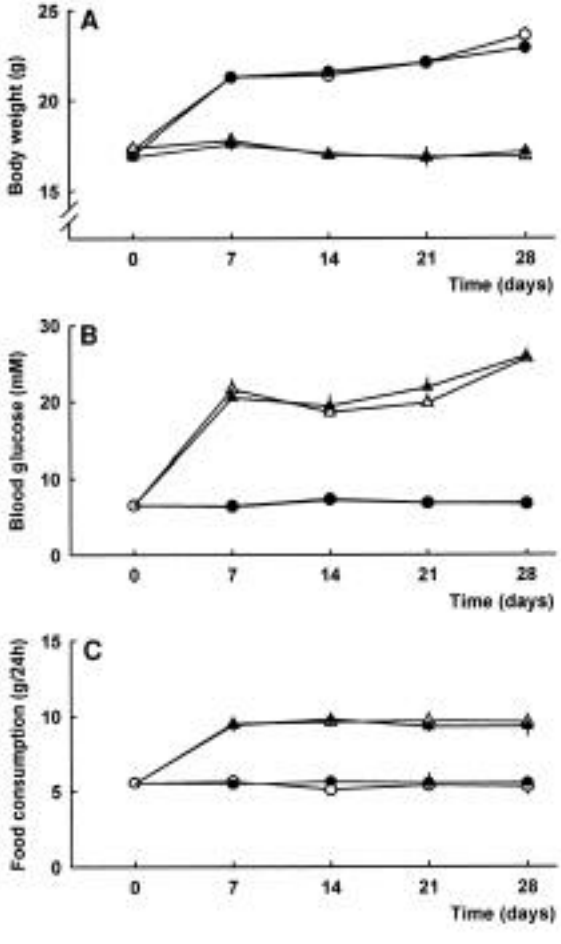

FIG. 1. Changes over 4 weeks in body weight (*A*), blood glucose (*B*), and food consumption (*C*) in nondiabetic animals treated with placebo (△) or G120K-PEG (2 mg/kg body wt every 2nd day) (▲) and diabetic animals treated with placebo (○) or G120K-PEG (●). Values are means ± SE; *n* = 16 in each group.

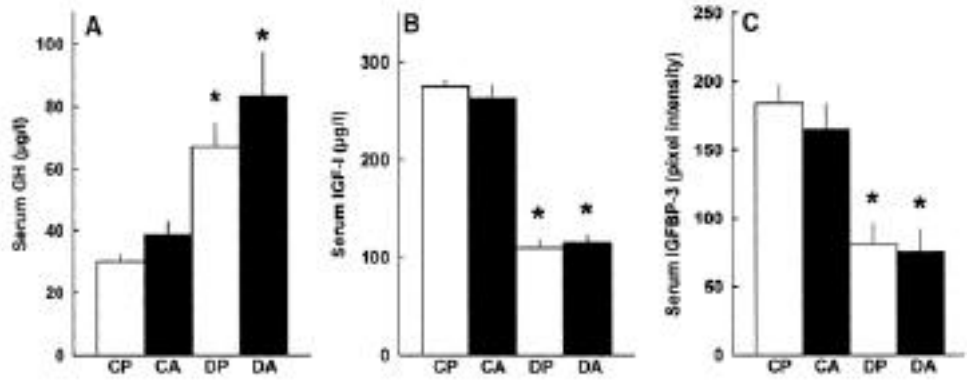

FIG. 2. Mean serum GH (*A*), IGF-I (*B*), and IGFBP-3 (*C*) levels at day 28 in nondiabetic mice treated with placebo (CP) or G120K-PEG (CA) and diabetic mice treated with placebo (DP) or G120K-PEG (DA). Values are means ± SE; n = 16 in each group. *P < 0.01 vs. nondiabetic control groups (CP and CA).

animals (Fig. 1*B*). Food consumption, expressed as g/24 h, was increased by ~80–90% in both diabetic groups when compared with nondiabetic groups (Fig. 1*C*), and G120K-PEG treatment did not affect the food intake in either diabetic or nondiabetic animals.

Serum GH, IGF-I, IGFBP-3, kidney IGF-I, and liver IGF-I. Figure 2 shows serum GH, IGF-I, and IGFBP-3 levels in the four experimental groups at the end of the study (i.e., day 28). It has been shown previously that barbital anesthesia induces a marked rise in GH levels that lasts for up to 90 min (33), and accordingly, the endogenous GH levels given in Fig. 2*A* are stimulated values. Increased serum GH levels amounting to ~120% (P < 0.05) above the level in nondiabetic animals were seen in response to the diabetic state per se (Fig. 2*A*). No effect of G120K-PEG administration was seen in any of the groups. Both serum IGF-I and IGFBP-3 decreased by ~60% (P < 0.01) in the diabetic groups, with no effect of G120K-PEG in nondiabetic or diabetic animals (Fig. 2*B* and *C*). The level of G120K-PEG ranged from 1,200 to 5,800 (mean 3,675) µg/l in nondiabetic animals and from 1,600 to 6,300 (mean 3,906) µg/l in diabetic animals.

Kidney IGF-I and liver IGF-I levels at day 28 are given in Table 1. Kidney IGF-I was increased in the untreated diabetic group when compared with nondiabetic control animals. This increase was fully abolished in G120K-PEG–treated diabetic animals. No effect of G120K-PEG treatment was seen in nondiabetic animals. Liver IGF-I levels were decreased in both diabetic groups when compared with their respective control groups, with no effect of G120K-PEG in nondiabetic or diabetic animals.

Kidney weight and glomerular volume. In Fig. 3, the kidney weights at day 28 are given for the four experimental groups. The kidney weight increase in the untreated diabetic group amounted to 11% when compared with that in nondiabetic control animals (163.0 ± 2.5 vs. 147.1 ± 2.1 mg, P < 0.01). The kidney weight increase was fully abolished in the G120K-PEG–treated diabetic animals, while no effect of treatment was seen in nondiabetic animals (Fig. 3*A*). When kidney weight was expressed relative to body weight, kidney weight in the untreated diabetic animals was 55% larger compared with that in nondiabetic control animals (9.69 ± 0.17 vs. 6.25 ± 0.11 mg/g body wt, P < 0.01), with a partial inhibition of the diabetes-associated renal enlargement in G120K-PEG–treated diabetic animals (8.46 ± 0.17, P < 0.01) (Fig. 3*B*). The increase in glomerular volume of untreated diabetic animals amounted to 25% when compared with that in nondiabetic control animals (1.52 ± 0.07 vs. 1.22 ± 0.06 10^5µm^3, P < 0.01), while the glomerular volume of G120K-PEG–treated mice (1.20 ± 0.05 10^5µm^3) was comparable to the two nondiabetic groups and significantly lower than that in untreated diabetic animals (P < 0.01) (Fig. 4*A*).

UAE. The 24-h UAEs in the four experimental groups are depicted in Fig. 4*B*. Untreated diabetic animals exhibited a marked elevation in UAE 28 days after induction of diabetes, amounting to 19.1 ± 1.3 vs. 1.5 ± 0.1 µg/24 h (P < 0.01) in nondiabetic mice (Fig. 4*B*). The UAE in the G120K-PEG–treated diabetic group was 57% lower (8.2 ± 1.1 µg/24 h, P < 0.01) when compared with the untreated diabetic mice, but it was still higher than that in the two nondiabetic control groups (Fig. 4) (P < 0.01). G120K-PEG treatment did not affect diurnal urine production in either nondiabetic or diabetic animals (data not shown).

DISCUSSION

The major new finding of the present study is specific renal effects of an exogenously administered GHR inhibitor in diabetic mice. Similar results have been published previously with long-acting somatostatin analogs (e.g., octreotide and lanreotide), which are believed to be less specific inhibitors of the GH/IGF axis (9,13–18). By recognizing the potential role

TABLE 1
Mean kidney IGF-I and liver IGF-I levels at day 28 in diabetic and nondiabetic mice treated with placebo or G120K-PEG

	Nondiabetic animals		Diabetic animals	
	Placebo (CP)	G120K-PEG (CA)	Placebo (DP)	G120K-PEG (DA)
Kidney IGF-I (ng/g tissue)	137 ± 4	135 ± 3	154 ± 6*	130 ± 6
Liver IGF-I (ng/g tissue)	44 ± 5	42 ± 4	28 ± 2†	24 ± 4†

Data are means ± SE; n = 6 in each group. *P < 0.05 DP versus all other groups (CP, CA, and DA); †P < 0.05 vs. CP and CA groups.

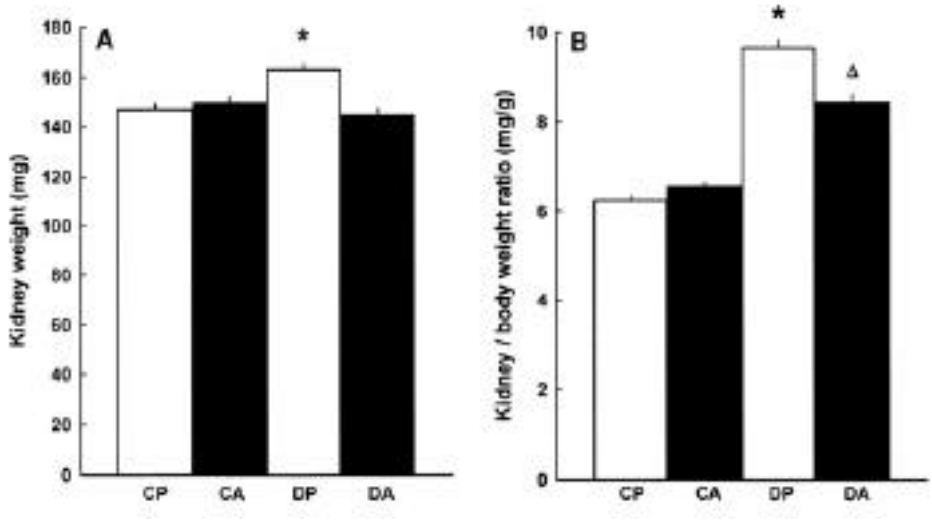

FIG. 3. Mean absolute kidney weight (*A*) and kidney weight:body weight ratio (*B*) at day 28 in nondiabetic mice treated with placebo (CP) or G120K-PEG (CA) and diabetic mice treated with placebo (DP) or G120K-PEG (DA). Values are means ± SE; $n = 16$ in each group. *$P < 0.01$ vs. all other groups (CP, CA, and DA); $^{\Delta}P < 0.01$ vs. nondiabetic groups (CP and CA).

that GH and IGFs may play in various pathophysiological conditions, including diabetic nephropathy, a series of highly specific antagonists of the GH action has been developed recently for potential therapeutic use. Initially, it was shown that alteration of single amino acids in the third α-helix of bovine (b)GH (residues 109–126) would result in a GH antagonist (26,27,34–36). In vitro experiments showed that the group of GH antagonists binds to the GHR with the same affinity as native GH, but in vivo, a phenotypic dwarf animal characterized by low circulating IGF-I levels and a proportional body composition develops when the GH antagonist is expressed in transgenic mice (34–36).

Recently published studies have described renoprotective effects of GH antagonists in long-term diabetic transgenic mice that express GH antagonists (bGH-G119R and hGH-G120R) (37–39). Compared with transgenic diabetic mice expressing wild-type bGH or bGH, diabetic mice expressing GH antagonists showed lesser glomerular damage (37,38), no increase in total urine protein (37), no glomerular hypertrophy (39), and no increase in glomerular α1 type IV collagen mRNA levels (39). Interestingly, the inhibitory effects of GH antagonists in transgenic mice were seen without alteration in glycemic control, as similar levels in blood glucose and HbA_{1c} were seen between the different diabetic animals expressing wild-type bGH, GH antagonists, or bGH (37,38). These studies support the hypothesis that GH and IGFs are involved in the pathogenesis of diabetic kidney disease in experimental diabetes. Theoretically, however, mice transgenic to GH antagonist may be less susceptible to diabetic renal changes because of an effect of GHR blockade and low circulating IGF-I levels (reaching dwarf levels) before the induction of diabetes. Further, the renal effects may be mediated indirectly through decreased circulating IGF-I levels per se at the time of diabetes induction. To further elucidate the potential usefulness of the GH antagonists as a therapeutic agent in terms of tolerance and specificity, the actual study was performed with exogenous administration of a long-acting formula of a GH antagonist (G120K-PEG) to diabetic and nondiabetic animals.

The present study demonstrates normalization of diabetic renal and glomerular hypertrophy along with a reduction in the diabetes-associated increase in UAE in diabetic mice treated with G120K-PEG for 1 month. The animals received G120K-PEG subcutaneously every 2nd day to maintain high diurnal levels. No toxic effects of G120K-PEG treatment were observed, as treated diabetic and nondiabetic mice had similar changes in food consumption, metabolic control, and body weight when compared with their respective placebo-treated groups.

Experimental diabetes in rats is characterized by suppressed serum levels of GH with loss of the characteristic pulsatility (40,41), while poorly controlled diabetes in humans is

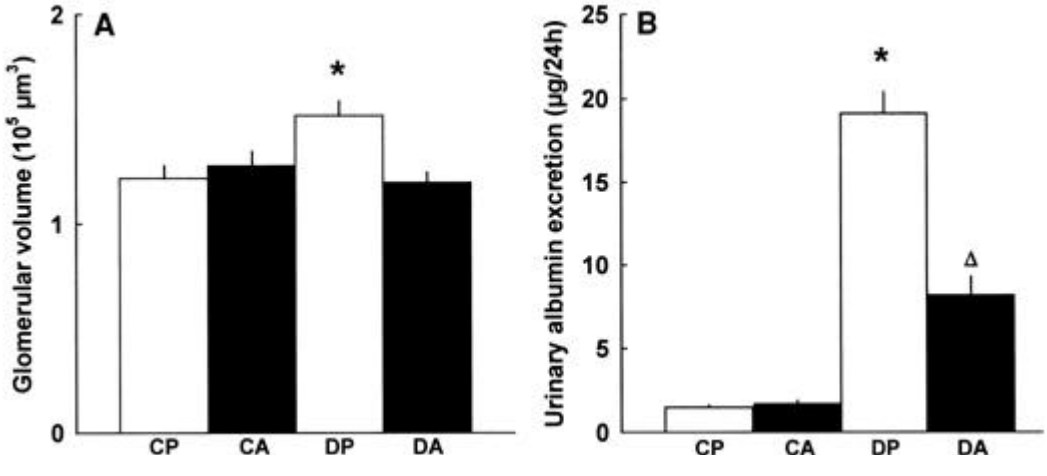

FIG. 4. Mean glomerular volume (*A*) and UAE (*B*) at day 28 in nondiabetic mice treated with placebo (CP) or G120K-PEG (CA) and diabetic mice treated with placebo (DP) or G120K-PEG (DA). Values are means ± SE; $n = 10$ in each group. *$P < 0.05$ vs. all other groups (CP, CA, and DA); $^{\Delta}P < 0.05$ vs. all other groups (CP, CA, and DP).

characterized by GH hypersecretion (42–44). However, in the present study, we found elevated barbital-stimulated GH levels in STZ-induced diabetic mice when compared with nondiabetic control mice. These findings are in concert with recent results obtained in NOD mice (45) and thus indicate that diabetic mice may be a better model, compared with diabetic rats, of the pertubations in the circulating GH/IGF levels in diabetes. There is support for the contention that the difference between experimental diabetes in rats and humans, with respect to the GH/IGF axis, may be restricted to GH. Accordingly, identical changes have been reported for the other elements in the axis in poorly controlled diabetic rats and humans, including changes in circulating levels of GHBP, IGF-I, and IGFBPs (7,9). Decreased circulating GHBP levels and decreased hepatic GHR number are well described features in experimental diabetes in rats (46–48). In contrast, however, few data have been published on the renal expression of GHR and GHBP in experimental diabetes. In one study, GHR mRNA expression was measured 4 days after induction of diabetes with STZ in rats, and unchanged levels were reported, despite decreasing levels in hepatic GHR mRNA (48). In a recent study including both short- and long-term diabetic rats, differential changes in kidney GHR and GHBP mRNA were observed (9). In the cortex, no change was seen in the GHR mRNA throughout the observation period of 6 months, while a significant increase in the GHBP mRNA and peptide was observed 1 month after induction of diabetes and was sustained for the rest of the study period (9). No changes were seen in GHR or GHBP mRNA in the medullary regions (9). These data indicate that although the GHR and GHBP mRNAs originate from the same gene, they are differentially regulated during the development of experimental diabetic kidney disease. Furthermore, these data imply a possible specific functional role for GHBP. Whether the increase in renal GHBP mRNA and peptide actually enhances renal GH availability to the GHR and thereby enhances a pathophysiological role of GH is still unknown. However, in the present study, renal effects of specific GHR blockade were seen without detectable changes in circulating endogenous GH, IGF-I, or IGFBP-3 levels, indicating a specific effect of G120K-PEG through a blockade of the renal GHR. This is further supported by the observation that the rise in kidney IGF-I in untreated diabetic animals was fully abolished by G120K-PEG treatment. Further, the liver IGF-I levels were unaffected by G120K-PEG treatment.

Theoretically, treatment with a GHR-blocking agent in diabetes could have a deteriorating effect on metabolism. Poorly controlled diabetes in humans and in mice (as shown in this study) present the paradox that hyperglycemia and insulinopenia do not suppress but stimulate GH secretion. Traditionally, the majority of circulating IGF-I has been considered to be synthesized in the liver, mainly under the control of circulating GH, and thus, in hyperglycemic diabetic subjects, serum IGF-I is reduced in spite of elevated GH. To explain this phenomenon it has been hypothesized that the diabetic metabolic deterioration first decreases hepatic IGF-I formation and serum IGF-I levels, which then secondarily induce GH hypersecretion (5–7). The metabolic consequences of these alterations are a vicious circle, where the hyperglycemia/insulinopenia-induced fall in serum IGF-I and rise in GH make optimal metabolic control more difficult to achieve. Accordingly, a potential negative effect of GH antagonists in diabetes could be a further lowering of circulating IGF-I levels with an associated deterioration of metabolic control. However, with the dose of GHR antagonist used in the present study, no such effects on metabolic control or endogenous GH/IGF-I levels were seen.

In conclusion, chronic administration of a GHR antagonist in diabetic mice has inhibitory effects on diabetic renal/glomerular hypertrophy and UAE without affecting metabolic control or circulating levels of GH, IGF-I, or IGFBP-3. Accordingly, the mechanism of the renal effects of the GHR antagonist may be mediated through an inhibitory effect on renal IGF-I through the renal GHR. The present study demonstrates the central role of the GH/IGF axis 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.

ACKNOWLEDGMENTS

This study was supported by grants from the Danish Medical Research Council (#9700592), the Danish Kidney Foundation, the Ruth König Petersen Foundation, the Novo Foundation, the Aage Louis-Hansen Memorial Foundation, the Nordic Insulin Foundation, the Eva and Henry Frænkels Memorial Foundation, and the Aarhus University-Novo Nordisk Center for Research in Growth and Regeneration (#9600822).

We are grateful to Karen Mathiassen, Kirsten Nyborg, and Ninna Rosenqvist for excellent technical assistance. This study is dedicated to the memory of E.M. Madsen.

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Author Queries (please see Q in margin and underlined text)