

Kidney Growth in Normal and Diabetic Mice Is Not Affected by Human Insulin-Like Growth Factor Binding Protein-1 Administration

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Insulin-like growth factor I (IGF-I) accumulates in the kidney following the onset of diabetes, initiating diabetic renal hypertrophy. Increased renal IGF-I protein content, which is not reflected in messenger RNA (mRNA) levels, suggests that renal IGF-I accumulation is due to sequestration of circulating IGF-I rather than to local synthesis. It has been suggested that IGF-I is trapped in the kidney by IGF binding protein 1 (IGFBP-1). We administered purified human IGFBP-1 (hIGFBP-1) to nondiabetic and diabetic mice as three daily sc injections for 14 days, starting 6 days after induction of streptozotocin diabetes when the animals were overtly diabetic. Markers of early diabetic renal changes (i.e., increased kidney weight, glomerular volume, and albuminuria) coincided with accumulation of renal cortical IGF-I despite decreased mRNA levels in 20-day diabetic mice. Human IGFBP-1 administration had no effect on increased kidney weight or albuminuria in early diabetes, although it abolished renal cortical IGF-I accumulation and glomerular hypertrophy in diabetic mice. Increased IGF-I levels in kidneys of normal mice

receiving hIGFBP-1 were not reflected on kidney parameters. IGFBP-1 administration in diabetic mice had only minor effects on diabetic renal changes. Accordingly, these results did not support the hypothesis that IGFBP-1 plays a major role in early renal changes in diabetes. *Exp Biol Med* 230:135–143, 2005

Key words: diabetes; growth hormone; insulin-like growth factor I; insulin-like growth factor binding protein 1; mouse

Long-term diabetes is accompanied by diabetic nephropathy in 15%–20% of patients with type 1 diabetes, and diabetic nephropathy is one of the most frequent causes of end-stage renal failure in developed countries (1). Increased kidney size, glomerular volume, and hyperfiltration are followed by an increase in urinary albumin excretion, mesangial proliferation, and accumulation of extracellular matrix. Progressive glomerular sclerosis leads to proteinuria and progressive renal insufficiency. The involvement of the growth hormone (GH)/insulin-like growth factor (IGF) axis in diabetic renal complications has been recognized for a long time (2).

IGF-I is a multifunctional polypeptide produced under the influence of pituitary GH (3). The liver produces most of the circulating IGF-I, although physiologically important autocrine and paracrine production occurs within other tissues. The biological functions of IGF-I are mediated by cell surface receptors, particularly by the IGF-I receptor (IGF-IR, also known as the Type 1 IGF receptor). Six high-affinity IGF binding proteins (IGFBP-1 to IGFBP-6) modulate the actions of IGF-I (4). Circulatory IGFBPs carry the major portion of serum IGF-I, neutralizing its hypoglycemic potential, prolonging its half-life, and transporting it to target tissues. Locally synthesized IGFBPs

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modulate the biological effects of IGF-I at the receptor level. In addition, IGFBPs may also have ligand-independent effects (4).

Morphologic and hemodynamic renal changes that occur in the insulinopenic diabetes model produced by streptozotocin (STZ), reflect the changes observed in the course of type 1 diabetes in humans. Following the onset of diabetes, IGF-I accumulates in the kidney, preceding the initiation of renal hypertrophy (5–7). An increased kidney IGF-I protein content, along with unchanged or decreased IGF-I messenger RNA (mRNA) levels, suggest that renal IGF-I accumulation is due to sequestration of circulatory IGF-I rather than to local synthesis (6).

IGF-I may get trapped in the kidney as a consequence of changes in renal IGFBPs. IGFBP-1 is considered a strong candidate both in capturing IGF-I locally and in mediating kidney growth *per se*. IGF-I and IGFBP-1 colocalize in the same portions of the nephron, in the same cell types, supporting the view that the actions of IGF-I may be modified by IGFBP-1 (8). In early STZ-induced diabetes in rats, cortical IGFBP-1 mRNA is increased (9).

Recently, we reported increased kidney weights after human IGFBP-1 (hIGFBP-1) administration in pituitary-deficient Snell dwarf mice. In addition, hIGFBP-1 coadministered with IGF-I neutralized the stimulating effects of IGF-I on body length and weight, but not on kidney weight (10). These results, suggesting an important role for IGFBP-1 in kidney enlargement, led us to investigate the effects of hIGFBP-1 administration on kidney parameters and regulation of the GH/IGF axis in a diabetic mouse model.

Materials and Methods

Human IGFBP-1 Isolation and Purification. Human IGFBP-1 was isolated from midgestational amniotic fluid obtained for diagnostic purposes with approval of the Ethics Committee of Sophia Children's Hospital, Rotterdam, The Netherlands, and purified on a Resource RPC 3-ml column connected to a fast protein liquid chromatography (FPLC) System (Automatic FPLC; Pharmacia Biotech, Uppsala, Sweden) as described in detail previously (11).

Animals and Procedures. Adult female NMRI mice aged 8 weeks (Bomholtgaard, Ry, Denmark) with initial body weights of 21.3 ± 0.9 g were used. Mice were housed five to six per cage and had free access to standard chow (Altromin 1324; Lage, Germany) and tap water. They were kept at constant temperature ($22^\circ \pm 2^\circ\text{C}$) and humidity ($55\% \pm 2\%$), under a 12:12-hr light:dark cycle (0600–1800 hrs). The study complied with Danish regulations for handling laboratory animals.

The animals were randomized into four groups, two of which were made diabetic: (i) control/placebo (CP, $n = 10$), (ii) control/hIGFBP-1 (CI, $n = 10$), (iii) diabetic/placebo (DP, $n = 15$), and (iv) diabetic/hIGFBP-1 (DI, $n = 15$). After

12 hrs of food deprivation (all groups), diabetes was induced at Day 0 by a single iv tail-vein injection of STZ (≈ 225 mg/kg body weight) dissolved in 0.154 M NaCl (pH 4). The animals were weighed, blood glucose levels were measured in tail-vein blood (Precision Xtra; Abbott Laboratories, MediSense Products, Bedford, MA), and urine was examined for glucose and ketone bodies (Neostix-4; Ames, Stoke Poges, Slough, UK) every second day throughout the study. Mice with ketonuria on Day 3 were treated with sustained release insulin implants (Linbit for mice; Linshin Canada, Inc., Scarborough, ON, Canada) to correct ketonuria and prevent massive body weight loss. Mice with blood glucose levels greater than 16 mM and without ketonuria were included in the study.

Human IGFBP-1 administration was initiated at Day 6, when the animals developed overt diabetes. Human IGFBP-1 was administered by three daily sc injections (at 0800, 1600, and 2400 hrs) for 14 days (0.3 mg hIGFBP-1/animal/day, based on previous study; Ref. 10), while placebo groups received an equivalent volume of vehicle alone (0.154 M NaCl) in the same time intervals (Days 6–19). Food consumption was measured per cage and expressed per mouse. Animals were sacrificed at Day 20 after sodium barbital (10 mg/kg body weight) ip anesthesia, exactly 2 hrs after the last hIGFBP-1 injection. Blood was drawn 5 mins later from the retro-orbital venous plexus. Serum was stored at -80°C until analysis. Immediately after extraction, kidneys and liver were weighed, snap-frozen in liquid nitrogen, and stored at -80°C . The middle part of the left kidney (2 mm thick) containing the papilla was fixed and embedded in paraffin for morphological measurements and immunohistochemistry.

Immunoassays. Serum GH, serum IGF-I, and renal cortical IGF-I levels were measured by radioimmunoassay as described previously (12). Serum insulin was measured by an ultrasensitive rat insulin enzyme-linked immunosorbent assay (DRG Diagnostics, Marburg, Germany). Semilog linearity of mouse serum and rat insulin was found at multiple dilutions, indicating antigen similarity between mouse and rat insulin. The intraassay and interassay coefficients of variation were $<5\%$ and $<10\%$, respectively.

Western Ligand Blotting (WLB) for Determination of Serum and Kidney IGFBPs. Serum samples (2 μl per lane) or tissue homogenates (200 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and WLB analysis of serum and renal cortical IGFBPs was executed as described previously (13). The relative densities of the bands were expressed in pixels.

Western Immuno Blotting (WIB) for IGFBP-1 and IGFBP-2. Serum samples (2 μl per lane) were separated by SDS-PAGE (15% polyacrylamide) and transferred by electroelution onto nitrocellulose membrane (Schleicher & Schuell, Munich, Germany). The blots were blocked for 12 hrs at 4°C in 5% nonfat milk and incubated overnight at 4°C with a polyclonal goat IGFBP-1 antibody

(Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1/1000, or with a polyclonal goat IGFBP-2 antibody (Santa Cruz Biotechnology) in a dilution of 1/1000. The IGFBP-1 antibody cross-reacts both with human and mouse IGFBP-1 (mIGFBP-1). After washing in Tris-buffered saline-Tween (0.1%), the membranes were incubated with an anti-goat IgG antibody conjugated with horseradish peroxidase (Pierce, Rockford, IL) for 1 hr at room temperature in a dilution of 1/12,000. The bands were visualized by chemiluminescence (BioWest; UPV Inc., Upland, CA) and quantified by a UPV BioImaging System (UPV Inc.).

Immunohistochemistry. The middle part of the left kidney (2 mm thick) containing the papilla was fixed in neutral buffered 4% paraformaldehyde and embedded in paraffin. Four-micrometer-thick sections were prepared by standard techniques and mounted on SuperFrost Plus slides (Fisher Scientific, Fairlawn, NJ). Tissue sections were then deparaffinized and rehydrated. Kidney sections were incubated with a polyclonal antibody against human IGF-I (a kind gift from Dr. Peter Gluckman, Liggins Research Institute, Auckland, New Zealand; 14) for 24 hrs at 4°C. The immunoreactivity was visualized by the avidin-biotin-peroxidase technique, using diaminobenzidine as the chromagen (Vectastain; Vector Laboratories, Burlingame, CA). The tissue sections were then counterstained with Carazzi's hematoxylin, dehydrated, and mounted with Permout (Fisher Scientific).

Real-time Polymerase Chain Reaction (PCR). Total RNA was extracted from cortical kidney and liver samples by Trizol Reagent following the instructions of the manufacturer (Gibco BRL-Life Technologies Inc., Gaithersburg, MD). Renal cortical and hepatic GH/IGF system gene expression was analyzed by means of real-time quantitative PCR (TaqMan), as described previously (11). Following complementary DNA synthesis, mRNA levels were quantitatively determined on an ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA) using SYBR-green technology. The primers used for mouse hypoxanthine phosphoribosyltransferase (mHPRT), mIGF-I, mIGFBP-1, mIGFBP-3, mouse growth hormone-receptor (mGHR), and mouse growth hormone binding protein (mGHBP) have been reported previously (11). The primer set used for mIGFBP-2 was forward primer, CGCGGGTACCTGTGAAAAGA and reverse primer, CCCTCAGAGTGGTCGTCATCA, resulting in an amplicon size of 77 base pairs (bp). The primer set used for mIGFBP-4 was forward primer, CATCCC-CATTCCAAACTGTGA and reverse primer, AC-CCCTGTCTTCCGATCCA, resulting in an amplicon size of 110 bp. The primer set used for mIGFBP-5 was forward primer, AGACCTACTCCCCCAAGGTCTT and reverse primer, TTTCTGCGGTCCTTCTTCACA, resulting in an amplicon size of 84 bp. The primer set used for mIGF-IR was forward primer, GGGATTTCTGCGCCAACA and reverse primer, CGTGGATGACGAAGCCATCT, resulting in an amplicon size of 62 bp.

PCR efficiencies for all primer sets were >95%. All measured values were normalized to mHPRT gene expression.

Estimation of Glomerular Volume. The middle part of the left kidney (2 mm thick) containing the papilla was fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate buffer, and embedded in paraffin. Sections (2 μ m) cut on a rotation microtome (Leica Rotation Microtome RM 2165; Leica, Vienna, Austria) were stained with periodic acid-Schiff and hematoxylin. The thickness of the sections was controlled routinely by a Digital Microcator ND 221 (Heidenhain, Traunreut, Germany) attached to the microscope. In each animal, the mean glomerular tuft volume (V_G) was determined from the mean glomerular cross sectional area (A_G) by light microscopy at a magnification of $\times 40$ as previously described (12). Profile areas were determined with a two-dimensional version of the nucleator (CAST; Olympus, Copenhagen, Denmark). A_G was determined as the average area of a total of 40 to 50 glomerular profiles (capillary tuft omitting the proximal tubular tissue within the Bowman capsule) and V_G was calculated from the following equation: $V_G = \beta/k \times (A_G)^{3/2}$, where $\beta = 1.38$ is the shape coefficient for spheres (the idealized shape of glomeruli), and $k = 1.1$ is a size distribution coefficient. The measurements were performed in a blinded fashion without the investigator's knowledge of the different groups.

Urinary Albumin and Creatinine Concentration. The urine samples were collected in the morning (0800–1100 hrs) by gentle massage over the lower part of the abdomen and stored at -20°C until analysis was performed. The urinary albumin concentration was determined by radioimmunoassay as previously described (12). The urinary creatinine concentration was measured by an automated technique adapted from the Jaffé reaction and corrected for the prevailing glucose content due to interference in the Jaffé reaction. The intraassay and interassay coefficients of variation were <5% and <10%, respectively, for both assays.

Statistical Analysis. Means were compared by one-way ANOVA followed by pairwise comparisons with the least significant difference method. Serum GH values <1.3 were assigned value 1.3, and four groups were tested by a Kruskal-Wallis test, followed by pairwise comparisons by a Mann-Whitney test (SPSS software package; SPSS Inc., Chicago, IL). Results are given as the mean \pm SEM, with n indicating the number of animals studied. Significance was assigned at $P \leq 0.05$.

Results

Body Weight and Metabolic Parameters. Body weights of mice from control groups steadily increased over the time of the study (Fig. 1A). Diabetic mice weighed significantly less than their respective control groups at all time points considered throughout the study, despite a

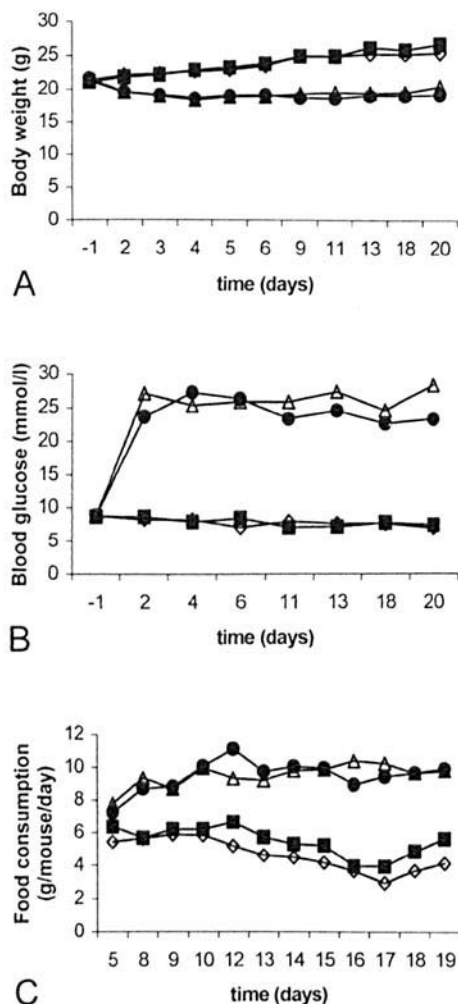


Figure 1. (A) Body weight, (B) blood glucose, and (C) food consumption in nondiabetic mice injected with placebo (\diamond) or hIGFBP-1 (\blacksquare) and diabetic mice injected with placebo (\triangle) or hIGFBP-1 (\bullet). Values are given as means ($n = 10$ for control, $n = 15$ for diabetic groups).

significantly higher daily food consumption (Fig. 1C). At Day 2, blood glucose levels in diabetic animals were approximately 3-fold higher than in controls and remained elevated throughout the study period (Fig. 1B). Insulin levels at Day 20 were lower in both diabetic groups compared with their respective controls (Fig. 2C). Injected hIGFBP-1 did not have any influence on mentioned parameters in the control and diabetic groups. Blood glucose levels were significantly decreased in diabetic mice administered hIGFBP-1 only in the last time point measured ($P = 0.016$).

Serum GH, IGF-I, and IGFBPs. In diabetic animals, serum GH levels were significantly increased (Fig. 2A) and IGF-I levels were decreased (Fig. 2B). Whereas hIGFBP-1 had no additional effect on GH serum levels, it further decreased IGF-I serum levels (Fig. 2A and B).

Serum levels of IGFBP-1, detected by WLB, were markedly higher in animals injected with hIGFBP-1, but

significantly less elevated in the diabetic group than in the control group (Fig. 2D). On WIB, injected hIGFBP-1 and endogenous mIGFBP-1 were clearly distinguishable by size (Fig. 2E). Mouse IGFBP-1 levels were not different between the groups. Circulating IGFBP-2 levels, detected both on WLB and WIB, were significantly elevated in diabetic animals receiving hIGFBP-1 compared with all other groups (Fig. 2D and E). Serum IGFBP-3 values were lower in diabetic animals compared with their respective controls, and were significantly reduced by hIGFBP-1 injections in both control and diabetic groups (Fig. 2D). Serum IGFBP-4 levels were decreased in both diabetic groups and were significantly increased in normal mice injected with hIGFBP-1 (Fig. 2D).

Kidney Weight, Glomerular Volume, and Urinary Albumin Excretion. Kidney weight is represented as the mean value of both kidneys (Fig. 3A). Kidneys of diabetic mice were significantly heavier than kidneys of their respective nondiabetic controls. Administration of hIGFBP-1, both in the control and diabetic groups, did not have any effect on kidney weight.

Glomerular volume was increased as a consequence of the diabetic state, and injection of hIGFBP-1 decreased it significantly (Fig. 3B). As an indicator of proteinuria the albumin:creatinine ratio (mg/mmol) was calculated. This ratio was increased in diabetic mice (Fig. 3C). The effect of hIGFBP-1 was not statistically significant.

Kidney IGF-I and IGFBPs. Extractable IGF-I protein content was increased in the renal cortex of diabetic mice receiving placebo (Fig. 4A). In diabetic mice receiving hIGFBP-1, IGF-I protein content was reduced (Fig. 4A). Human IGFBP-1 administration increased IGF-I levels in the kidneys of normal mice (Fig. 4A). Immunostainable IGF-I was clearly visible in both diabetic groups, but was absent in both nondiabetic groups. In diabetic kidneys, IGF-I was predominantly localized in papillary collecting ducts (Fig. 4C) and to some extent in cortical collecting ducts.

Renal cortical IGFBP-1 protein levels were markedly increased in groups receiving hIGFBP-1, and they were higher in diabetic group than in control group (Table 1 and Fig. 4B). IGFBP-2 protein content was higher in kidneys of diabetic animals receiving hIGFBP-1 compared with that of all groups (Table 1). Renal cortical IGFBP-3 protein levels were decreased in both diabetic groups (Table 1). The renal cortical IGFBP-4 protein level was decreased in diabetic mice receiving placebo, with an increase due to hIGFBP-1 administration (Table 1).

Expression of the GH/IGF Axis Genes in Kidney and Liver. Renal cortex IGF-I mRNA concentration was significantly reduced in both diabetic groups (Fig. 5). IGFBP-1 mRNA levels were increased in the control group receiving hIGFBP-1 and decreased in both diabetic groups (Table 1 and Fig. 5). Cortical expression of IGFBP-2 mRNA in kidney was not significantly different between the groups (Table 1). Renal mRNA expression of IGFBP-3 to

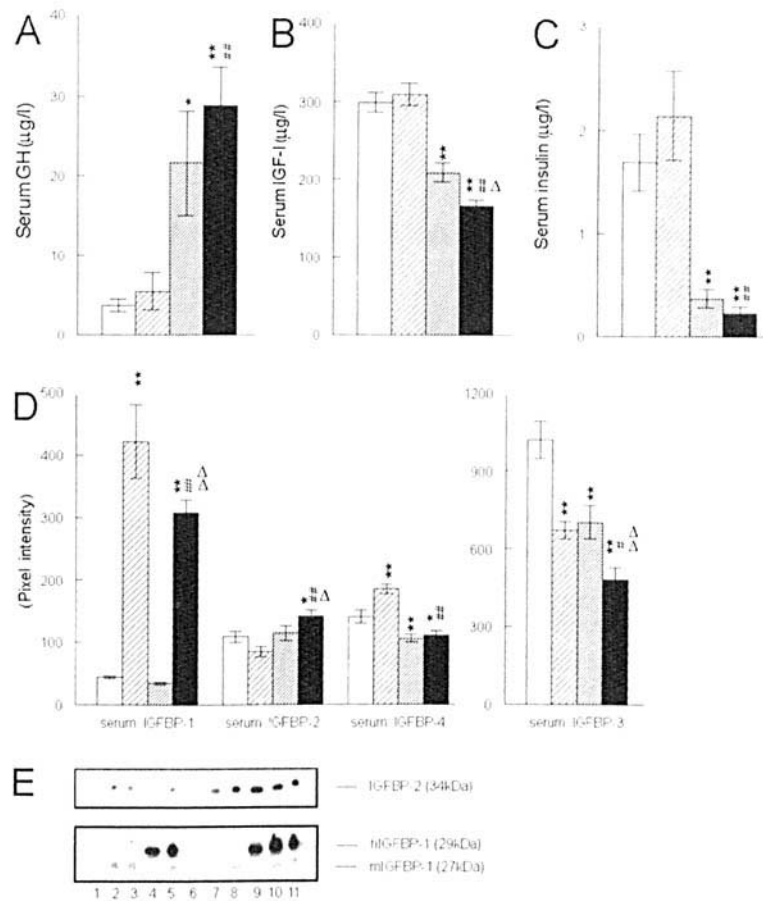


Figure 2. (A) Serum growth hormone (GH), (B) IGF-I, (C) insulin, and (D) IGFbps (WLB) levels at Day 20 in nondiabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars), and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means \pm SEM ($n = 10$ for control, $n = 15$ for diabetic groups). $\star\star P \leq 0.005$; $\star P \leq 0.05$ vs. CP group; $\#\# P \leq 0.005$; $\# P \leq 0.05$ vs. CI group; $\Delta\Delta P \leq 0.005$; $\Delta P \leq 0.05$ vs. DP group. Serum IGFBP-1 (hIGFBP-1, 29 kDa; mIGFBP-1, 27 kDa) and IGFBP-2 (34 kDa) by WB in CP (lanes 1–3), CI (lanes 4 and 5), DP (lanes 6–8), and DI (lanes 9–11) groups (E).

IGFBP-5 was decreased in both diabetic groups (Table 1). Levels of renal IGF-IR mRNA were significantly lower in all groups compared with the CP group ($P < 0.001$, data not shown). Expression of GH receptor mRNA was decreased in the control group receiving hIGFBP-1 ($P = 0.018$, CI vs. CP) and in the diabetic group receiving placebo ($P = 0.014$, DP vs. CP, data not shown). GH

binding protein mRNA levels were decreased in kidneys of both diabetic groups ($P \leq 0.001$, DP vs. CP and DI vs. CI, data not shown).

Hepatic IGF-I mRNA was down-regulated in diabetes and up-regulated in normal mice receiving hIGFBP-1 ($P \leq 0.005$, DP vs. CP; $P \leq 0.05$, DI vs. CI; $P \leq 0.05$, CI vs. CP, data not shown). Liver IGFBP-1 mRNA levels were

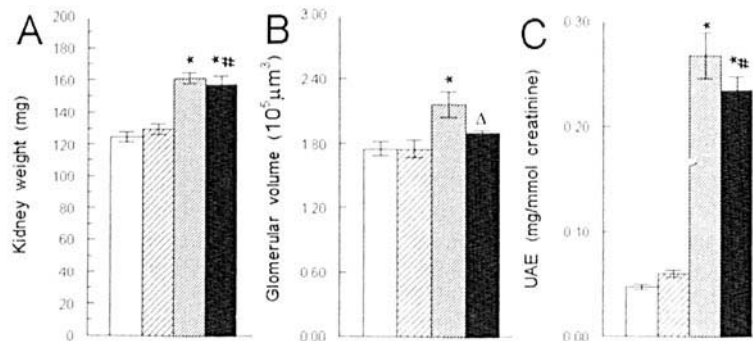


Figure 3. (A) Kidney weight, (B) glomerular volume, and (C) urinary albumin excretion at Day 20 in nondiabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means \pm SEM ($n = 10$ for control, $n = 15$ for diabetic groups). $\star P \leq 0.005$ vs. CP group, $\#\# P \leq 0.001$ vs. CI group, $\Delta P < 0.05$ vs. DP group.

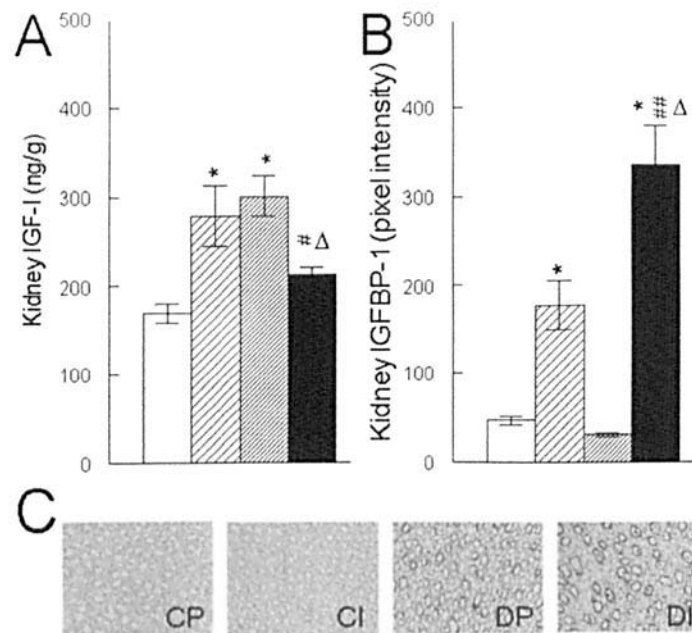


Figure 4. (A) Kidney IGF-I and (B) IGFBP-1 levels and immunostainable IGF-I in papillary collecting ducts ($\times 20$) (C) at Day 20 in nondiabetic mice injected with placebo (CP, white bars) or hIGFBP-1 (CI, light shaded bars) and diabetic mice injected with placebo (DP, dark shaded bars) or hIGFBP-1 (DI, black bars). Values are means \pm SEM [$n = 10$ for control, $n = 15$ for diabetic groups (A); $n = 9$ (B)]. $\star P \leq 0.005$ vs. CP group; $\#\# P \leq 0.005$; $\# P \leq 0.05$ vs. CI group; $\Delta P \leq 0.001$ vs. DP group.

significantly increased in diabetic animals receiving hIGFBP-1 compared with those in all groups ($P \leq 0.05$, data not shown). IGFBP-2 mRNA concentration was elevated in diabetic livers ($P < 0.001$, DP vs. CP; $P = 0.007$, DI vs. CI, data not shown). Hepatic IGFBP-3 mRNA was decreased in diabetic mice ($P = 0.001$, DP vs. CP) and restored to control levels when hIGFBP-1 was injected ($P = 0.013$, DI vs. DP, data not shown). Levels of IGFBP-4 and IGFBP-5 mRNA were not different between the groups (data not shown). Expression of GH receptor and GH-binding protein mRNA was decreased in livers of both diabetic groups ($P \leq 0.001$, DP vs. CP and DI vs. CI, data not shown).

Discussion

The major new finding of this study is that hIGFBP-1 administered for 2 weeks to mice with early STZ-diabetes had no further effect on the increase in kidney weight and albuminuria. However, hIGFBP-1 administration abolished the renal IGF-I accumulation and the increase in glomerular volume in diabetic animals.

The majority of previously published data on the involvement of the IGF system in diabetic renal hypertrophy have been obtained in the STZ-diabetic rat model, which is characterized by growth hormone hyposecretion (2). In contrast, as recently described, the STZ-diabetic mouse model is characterized by GH hypersecretion, thereby being

Table 1. Kidney IGFBPs mRNA and Protein Levels at Day 20 in Nondiabetic and Diabetic Mice Injected with Placebo or hIGFBP-1^a

Kidney	Messenger RNA ^b				Protein ^c			
	CP	CI	DP	DI	CP	CI	DP	DI
IGFBP-1	1 \pm 0.11	1.81 \pm 0.25 [*]	0.54 \pm 0.09 [*]	0.3 \pm 0.05 ^{**}	46.8 \pm 4.8	176.5 \pm 27.5 [*]	30.6 \pm 2.3	334.4 \pm 45.6 ^{**} , ^{***}
IGFBP-2	1 \pm 0.15	0.86 \pm 0.14	0.73 \pm 0.09	0.81 \pm 0.12	357.8 \pm 20.2	358 \pm 22	334.4 \pm 22	485.9 \pm 29 ^{**} , ^{***}
IGFBP-3	1 \pm 0.05	1.04 \pm 0.1	0.47 \pm 0.02 [*]	0.4 \pm 0.04 ^{**}	331.8 \pm 28.5	318.5 \pm 14.6	197.9 \pm 14.9	189.7 \pm 8 ^{**}
IGFBP-4	1 \pm 0.09	0.9 \pm 0.08	0.46 \pm 0.06 [*]	0.41 \pm 0.04 ^{**}	160 \pm 12	136.5 \pm 13.5	79.5 \pm 3 [*]	113.9 \pm 9.7 ^{***}
IGFBP-5	1 \pm 0.06	0.85 \pm 0.12	0.65 \pm 0.05 [*]	0.6 \pm 0.03 ^{**}				

^a CP, Nondiabetic mice injected with placebo; CI, nondiabetic mice injected with hIGFBP-1; DP, diabetic mice injected with placebo; DI, diabetic mice injected with hIGFBP-1. Values are means \pm SEM (mRNA, $n = 10$; protein, $n = 9$).

^b Artificial values, CP value set as 1.

^c Protein levels are presented as pixel intensity.

^{*} $P \leq 0.05$ vs. CP group; ^{**} $P \leq 0.05$, vs. CI group; ^{***} $P \leq 0.05$ vs. DP group.