Effects of experimental diabetes on renal IGF/IGFBP system during neonatal period in the rat

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De La Puente, A., L. Goya, S. Ramos, M. A. Martín, C. Alvarez, F. Escrivá, and A. M. Pascual-Leone. Effects of experimental diabetes on renal IGF/IGFBP system during neonatal period in the rat. Am J Physiol Renal Physiol 279: F1067–F1076, 2000.—Changes in the renal synthesis and concentration of insulin-like growth factors (IGFs) and their serum-binding proteins (IGFBPs) reported in insulin-deficient diabetes mellitus may be implicated in the alterations of the kidney function and morphology accompanying this disease. Most research on this subject has been carried out in adult animals, as well as in peripubertal rats, when the regulation of the IGF system is fully dependent on serum growth hormone (GH). However, relevant differences in the regulatory pathways of IGFs between adult and neonatal periods have been described. To examine the response of the IGF/IGFBP system of neonatal kidney to diabetes, renal IGF-I and -II and IGFBP-1, -2, and -3 concentration and mRNA expression were determined in streptozotocin-induced diabetic rat neonates. Diabetic neonates exhibited a kidney weight-to-body weight ratio higher than that of control rats, together with decreased IGF-I and increased IGF-II renal concentration. Because kidney mRNA expression of both IGFs decreased, the elevated renal IGF-II might result from increased uptake from circulation. Insulin treatment recovered the altered IGFs to control values, indicating the insulin-dependent regulation of IGFs in the neonatal kidney. Elevated levels of the IGFBP-1 and -2 in the kidney of diabetic neonates did not result from changes in their kidney mRNA transcript expression, suggesting also a possible uptake from circulation.

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THE GROWTH HORMONE (GH)/insulin-like growth factor (IGF)/IGF binding protein (IGFBP) system is involved in the development of the kidney (14). In adults, increased GH levels due to elevated endogenous secretion or pharmacological administration causes an increase in renal mass (14). The systemic induction of IGF-I by GH most likely mediates much of the GH effects on glomerular hyperthrophy and, in concert, transgenic mice overexpressing IGF-I have increased kidney and nephron size (10). However, in juvenile rats the rapid compensatory renal growth after unilateral nephrectomy appears to be GH independent (31). On the other hand, the most intriguing physiological events involving the IGF system in rats is that the fetal serum profile, characterized by high IGF-II and IGFBP-2, is replaced around the third week of life by the adult-type profile of high IGF-I and IGFBP-3, with a dramatic reduction in IGF-II and IGFBP-2 (12). Most of the studies on the renal IGF/IGFBP system have been carried out by investigating only the influence of the effects of IGF-I on growth and function of the kidney (14, 15). However, in weaning rats renal IGF-II levels increase transiently (38) and may contribute to compensatory nephron growth. It remains unclear whether there are different mechanisms between juvenile and adult rats by which GH and IGFs contribute to renal growth.

Diabetic nephropathy, one of the major complications of diabetes mellitus, is manifested by an increased kidney size in both humans (34, 42) and rats (39, 40). The possible role of the IGF/IGFBP system has been studied in a number of different animal models (14), particularly in the insulinopenic diabetes model in the rat induced by the β-cell toxin streptozo- tocin (STZ). Insulin-like growth factor-I (IGF-I) is a mitogenic polypeptide with important effects on cultured mesangial cells (8), and IGF-I receptors are found in kidney membranes, glomerular mesangial cells, and tubules (1, 33). Six well-characterized IGF binding proteins modulate the biological actions of the IGFs (35). Serum IGFBPs bind the major portion of IGFs in the serum, protect them from proteases, and transport the IGFs out of the circulation to their target tissues. These IGFBPs may either potentiate or inhibit the local effects of IGF-I, but they have independent biological effects (35).

Changes in the renal IGF system induced by diabetes have been mostly studied at adult stages (2, 6, 17, 41), and less frequently in rats at pre- and postpubertal stages (3, 5, 32). Epidemiological studies have suggested that diabetes-associated renal changes are less

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common in young patients than in adults (9, 30); however, to date there are no reports on the alterations of the renal IGFs system that occur in a diabetic situation during the early stages of extrauterine life. Although diabetic nephropathy has never been described in neonatal rats, the changes observed in the diabetic kidney yield a good experimental model to investigate the influence of the IGF/IGFBP system on renal growth during the neonatal period. We have previously shown that the developmental pattern for the IGF/IGFBP system is clearly disturbed in diabetic rats, where high serum glucose levels and low serum insulin seem to play a major role in IGF and IGFBP regulation at stages when GH is not the main regulatory factor (21, 36). Because an age-dependent, differential regulation of serum and liver IGFs and IGFBPs in diabetes has been observed (21, 36), our goal was to investigate the diabetes-induced alterations of the IGF/IGFBP system in the neonatal kidney, to evaluate potential differences in the renal IGF regulation between adult and perinatal stages. To this end, we measured the early changes in the most important components of the IGF/IGFBP system in the kidney after induction of diabetes by STZ in neonatal rats at 8, 14, and 22 days of life and further recovery by insulin treatment. In this article, we report changes induced by experimental diabetes in the peptide concentration and mRNA expression of IGFs and IGFBPs in kidney, which seem different from those previously described in kidney of adult diabetic rats. These changes are discussed, together with those in liver and serum at the same stage of development as previously reported by our laboratory.

MATERIALS AND METHODS

Materials

Recombinant human IGF-I and II (Boehringer Mannheim, Leverkusen, Germany) were used as standard and for iodination. RNase A and RNase T1 were also purchased from Boehringer Mannheim. STZ was a kind gift of Upjohn (Kalamazoo, MI). Lente insulin was kindly supplied by Novo Nordisk Pharma (Madrid, Spain). BCP-Na and Hyperfilm-MP autoradiography film were obtained from Amersham (Amersham Ibérica, Madrid, Spain). Polyvinylidene fluoride (PVDF) immobilon-P Millipore membranes were purchased from ICN (Nuclear Ibérica, Madrid, Spain). Recombinant human IGF-I and II were labeled by a modified chloramine T method (36, 37). The specific activity achieved with this method was ~90–175 mCi/mg for both peptides. Before IGF-I and -II determinations, serum IGFBPs were removed by standard acid gel filtration. This method has proved to be the most reliable for use with neonatal rat serum (36, 37). All experiments were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH publication no. 85–23, revised 1985).

Animals and Experimental Models

Animals. Wistar rats bred in our laboratory with controlled temperature and an artificial dark-light cycle were used throughout the study. Control animals were fed a standard laboratory diet ad libitum. Water was given ad libitum. Blood was harvested from the trunk after decapitation of rats at 8, 14, or 22 days of life, allowed to clot on ice for 30 min, and serum was separated and stored at –80°C until assayed. Kidneys were frozen in liquid N2 on removal for IGF and IGFBP RNA extraction and IGF determination.

All experiments were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH publication no. 85–23, revised 1985).

Experimental models: STZ-induced diabetes and insulin treatment. Diabetes was induced by a single intraperitoneal injection of STZ (70 mg/kg body wt) in 0.05 M citrate buffer, pH 4.5, 4 days before the animals were euthanized (at 4, 10, and 18 days of life) and was confirmed by the determination of glycemia (Table 1) and insulinemia (see RESULTS) at the time of time of death (8, 14, and 22 days). To study insulin-induced recovery from diabetes, rats on days 6 and 14 were treated with STZ and, on days 10 and 18, respectively, 3 IU·100 g body wt -1·day -1 of Lente insulin were administered in two doses (0900 and 1800 ac), a treatment that kept glycemia within the normal euglycemic range until the time of death (days 14 and 22, respectively). Insulin treatment was not assayed in 8-day-old rats because of technical difficulties.

Glycemia and Plasma Glucose Determination

Serum glucose was measured with a Reflolux IIM (Boehringer Mannheim, Leverkusen, Germany) glucose analyzer. Plasma immunoreactive insulin was estimated with purified rat insulin as the standard (NOVO), an antibody to porcine insulin, which cross-reacted similarly with pork and rat insulin standards, and moniodinated 125I-labeled human insulin. The minimal detectable dose was 0.04 ng/ml, with a coefficient of variation within and between assays of 10%.

Serum IGF-I and -II Determinations

Recombinant human IGF-I and II were labeled by a modified chloramine T method (36, 37). The specific activity achieved with this method was ~90–175 mCi/mg for both peptides. Before IGF-I and -II determinations, serum IGFBPs were removed by standard acid gel filtration. This method has proved to be the most reliable for use with neonatal rat serum (36, 37). The RIA for IGF-I was as previously described (36, 37). The coefficients of variation within and between assay were 8.0 and 12.4%, respectively. Interference of IGF-II in the RIA for IGF-I was determined as the relationship between the ED50 of the displacement curves of both peptides and was 11.7%. IGF-I data were corrected for IGF-II interference.

Rat liver membrane radioreceptor assay for IGF-II was performed as described elsewhere (36, 37) with purified rat

Table 1. Glycemia of control and diabetic rats at 8 days of life and control, diabetic, and insulin-treated rats at 14 and 22 days of life

<table>
<thead>
<tr>
<th></th>
<th>8 Days</th>
<th>14 Days</th>
<th>22 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>113±9</td>
<td>155±8</td>
<td>144±23</td>
</tr>
<tr>
<td>Diabetic</td>
<td>355±125*</td>
<td>390±67*</td>
<td>397±106*</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>61±4†</td>
<td>64±12‡</td>
<td></td>
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</tbody>
</table>

Values are means ± SD, expressed as mg glucose/100 ml serum; n = 12–15, *P < 0.05 relative to age-paired controls. †P < 0.05 relative to age-paired diabetic rats.
Within- and between-assay coefficients of variation were 8.4 and 9.9%, respectively. IGF-I interference was 0.1%.

Western Ligand Blotting and Western Immunoblotting

Western ligand blots were performed as previously described (36, 37). Autoradiographs were quantified by two-dimensional densitometry by using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA). Western immunoblots for enhanced chemiluminescence were performed in PVDF immobilon-P membranes. PVDF membranes were blocked with 5% (wt/vol) nonfat dry milk for 60 min in Tris-buffered saline (TBS; 0.01 M Tris, 0.15 M NaCl, pH 8) with 0.05% Tween 20. Membranes were then incubated with a 1:100 dilution (as suggested by the manufacturer, Santa Cruz Biotechnology) of affinity-purified goat polyclonal anti-rat IGFBP-1 or rat IGFBP-2 in the same buffer (TBS-Tween plus 5% nonfat dry milk) at 4°C overnight, after which the membrane was washed three times for 10 min in TBS-Tween. After a 1-h incubation at room temperature with a 1:1,000 dilution of anti-goat IgG-horseradish peroxidase in TBS-Tween plus 5% nonfat dry milk, the membrane was washed three times with TBS-Tween and, finally, once with TBS alone. Antigen-antibody complexes were detected after enhanced chemiluminescence.

Preparation of RNA

Total RNA was prepared by homogenization of kidneys in guanidinium thiocyanate as originally described for liver (7). Samples were electrophoresed through 1% agarose, 2.2 M formaldehyde gels, and stained with ethidium bromide to visualize the 28S and 18S ribosomal RNA and thereby confirm the integrity of the RNA and normalize the concentration of RNA in the different lanes before loading. A pT7 RNA 18S antisense control template (Ambion, Austin, TX) was used for lane loading control.

Riboprobes

Rat IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 cDNAs were kindly provided by Dr. C. T. Roberts, Jr., and Dr. D. LeRoith (National Institutes of Health, Bethesda, MD). See Refs. 36 and 37 for details on the preparation of riboprobes for

Table 2. Organ weight-to-body weight ratio of control, diabetic, and insulin-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Kidney OW/BW (×1,000)</th>
<th>Liver OW/BW (×1,000)</th>
<th>Spleen OW/BW (×1,000)</th>
<th>Pancreas OW/BW (×1,000)</th>
<th>Brain OW/BW (×1,000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.5 ± 0.4</td>
<td>21.1 ± 1.5</td>
<td>5.9 ± 0.4</td>
<td>3.0 ± 0.4</td>
<td>32.9 ± 2.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>6.5 ± 0.4*</td>
<td>28.6 ± 1.7*</td>
<td>5.7 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>41.2 ± 2.4*</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>7.9 ± 0.5**†</td>
<td>35.4 ± 0.8**†</td>
<td>4.9 ± 0.6</td>
<td>4.2 ± 0.5</td>
<td>26.7 ± 1.8</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.7 ± 0.3</td>
<td>32.1 ± 1.1</td>
<td>4.6 ± 0.7</td>
<td>3.2 ± 0.5</td>
<td>28.1 ± 1.5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8.6 ± 0.6*</td>
<td>32.7 ± 2.4</td>
<td>6.0 ± 0.9</td>
<td>3.7 ± 0.4</td>
<td>28.2 ± 1.3</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>6.3 ± 0.6*</td>
<td>54.2 ± 5.2*</td>
<td>4.2 ± 0.4</td>
<td>5.2 ± 0.5</td>
<td>23.7 ± 2.8*</td>
</tr>
<tr>
<td>22 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.3 ± 0.3</td>
<td>37.6 ± 1.4</td>
<td>4.1 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>19.4 ± 1.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>6.5 ± 0.6*</td>
<td>39.5 ± 2.3</td>
<td>5.2 ± 0.6</td>
<td>4.1 ± 0.4</td>
<td>23.6 ± 1.6*</td>
</tr>
<tr>
<td>Insulin-treated</td>
<td>6.3 ± 0.6*</td>
<td>54.2 ± 5.2*</td>
<td>4.2 ± 0.4</td>
<td>5.2 ± 0.5</td>
<td>23.7 ± 2.8*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 12–15. OW, organ wt., BW, body wt. *P < 0.05 relative to age-paired controls. †P < 0.05 relative to age-paired diabetic rats.

Fig. 1. Kidney insulin-like growth factor (IGF)-I (A) and -II (B) concentration in diabetic neonatal rats. IGF-I and -II were assayed by RIA and radioreceptor assay (RRA), respectively, in kidney homogenates from control and diabetic rats at 8 and 14 days of life and control, diabetic, and insulin-treated rats at 22 days of life. Values are means ± SD of 8–10 different samples. P < 0.05 relative to control group at same age. P < 0.05 relative to diabetic group at same age.

liver plasma membrane. Within- and between-assay coefficients of variation were 8.4 and 9.9%, respectively. IGF-I interference was <0.1%.

Western Ligand Blotting and Western Immunoblotting

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Preparation of RNA

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Riboprobes

Rat IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 cDNAs were kindly provided by Dr. C. T. Roberts, Jr., and Dr. D. LeRoith (National Institutes of Health, Bethesda, MD). See Refs. 36 and 37 for details on the preparation of riboprobes for
IGF-I, -II, IGFBP-1, -2, and -3. The size of the protected fragments was 386 (IGF-Ib), 500 (IGF-II), 700 (IGFBP-1), 550 (IGFBP-2), and 343 bp (IGFBP-3). The pT7 RNA 18S antisense control template was incubated with T7 RNA polymerase to produce a 109-nucleotide runoff transcript, 80 nucleotides of which are complementary to human 18S ribosomal RNA. All the above riboprobes were synthesized with 800 Ci/mmol [32P]UTP.

Solution Hybridization/RNAse Protection Assay

Solution hybridization/RNAse protection assays were performed as previously described (36, 37). Briefly, 20 μg of total RNA from kidney were hybridized with 500,000 counts/min of the 32P-labeled riboprobes described above for 18 h at 45°C in 75% formamide and 400 mM NaCl. After RNAse digestion with a buffer containing 40 mg/ml RNAse A and 2 mg/ml RNAse T1 for 1 h at 37°C, protected RNA-RNA hybrids were resolved on denaturing 8% polyacrylamide and 8 M urea gels. Autoradiography was performed at −70°C against Hyperfilm MP film between intensifying screens. Bands representing protected probe fragments were quantified by using a Molecular Dynamics scanning densitometer and accompanying software.

Statistical Analysis

All data are presented as means ± SD. Statistical comparisons were performed by one-way ANOVA, followed by the protected least significant difference test (36, 37).

RESULTS

Serum Glucose and Insulin, Organ Weight-to-Body Weight Ratio, and Kidney IGF-I and -II Peptide Levels

To ensure that diabetes was established, serum glucose was measured 4 days after STZ administration; close to 90% of rats treated with STZ showed significant higher levels of glycemia than those untreated, but only rats with serum glucose values above 250 mg/dl were used for the experiments (Table 1). Treatment with insulin for 4 days reduced plasma glucose in diabetic rats to levels below those of control animals (Table 1). Random blood samples were assayed for insulin concentration to double-check the diabetic condition of the neonatal rats. Insulin concentration was lower in diabetic than in control rats in all cases (30.6 ± 7.3 vs. 97.4 ± 3.7 μU/ml insulin, P < 0.05). Because changes in the kidney weight-to-body weight ratio could reflect a reduction in total body weight by simple dehydration of the diabetic animal, other organ weight-to-body weight ratios were also calculated and are depicted in Table 2. When the data were submitted to ANOVA, relevant changes in the organ weight-to-body weight ratios were found in kidney and liver but not in spleen, pancreas, and brain. An increase in kidney weight-to-body weight ratio was found in
diabetic rats at 8, 14, and 22 days, and insulin treatment of the 14- and 22 day-old groups for 4 days did not recover this ratio (Table 2). An increase in liver weight-to-body weight ratio was observed in 8-day-old diabetic rats as well as in diabetic rats treated with insulin vs. control rats at 14 and 22 days of life (Table 2). Finally, an increase in brain weight-to-body weight ratio was observed in diabetic rats at 8 and 22 days of life. However, no sustained increase throughout postnatal growth was found in the organ weight-to-body weight ratios for liver, spleen, pancreas, and brain in the diabetic group.

Figure 1 shows the analysis of IGF-I and -II peptides by RIA and radioreceptor assay, respectively. IGF-I peptide levels in kidney of diabetic rats were reduced only at 22 days. IGF-II peptide levels in kidney of control rats decreased with age from day 8 to day 22 and, contrary to IGF-I, increased levels of IGF-II were found in kidney of diabetic rats at 8 and 22 days. In both cases, IGF-I and -II, values in insulin-treated rats recovered to levels of those in control rats.

**Kidney mRNA Expression of IGF-I and -II**

RNA expression levels of IGF-I and -II in kidneys of neonatal control, diabetic, and insulin-treated rats were determined by RNase protection assay of 20 μg of total RNA samples. IGF-I mRNA expression levels were found significantly decreased in diabetic rats vs. controls at all stages (8, 14, and 22 days of life) (Fig. 2). Treatment of diabetic rats with insulin for 4 days between days 18 and 22 provoked a significant increase in the reduced IGF-I mRNA levels over those in control rats at the same age (Fig. 2). IGF-II mRNA expression levels decreased with age in kidneys of control rats (Fig. 2); in a comparison of films with equal exposure times, IGF-II values were similar to those of IGF-I at 8 days, significantly lower at 14 days, and almost undetected by RNase protection assay (RPA) at 22 days (Fig. 2). As with IGF-I, IGF-II mRNA expression levels were found also reduced in neonatal diabetic rats vs. controls at 8 and 14 days, whereas no significant changes in the mRNA expression of kidney IGF-II were observed in diabetic and insulin-treated rats at 22 days. The figures depict representative experiments, and densitometric analysis of 3 different experiments (9 samples/group) confirmed all the above results (Fig. 2). As proof for gel loading normalization, the 18S band is shown below the IGFs bands.

To examine whether IGF-I and -II peptide levels in kidney reflected IGFs synthesis by the organ, peptide levels in nanograms per gram tissue were correlated with optical density in arbitrary units of mRNA expression bands. A good correlation between peptide levels and mRNA expression was observed for IGF-I (r = 0.7) and IGF-II (r = 0.75) in 8-, 14-, and 22-day-old control rats (Fig. 3). No correlation was found in diabetic animals (data not shown).

**Protein Concentration and mRNA Expression of IGFBPs in Kidney**

The amount of protein of the major binding proteins of IGFs, IGFBP-3 (45 kDa), IGFBP-1 and -2 (30 kDa), and IGFBP-4 (24 kDa) in kidney of control and diabetic neonatal rats was determined by Western ligand blot. No differences were found in the intensity of the 45-kDa band between control and diabetic rats at 8 and 14 days, but in diabetic kidneys at 22 days of life a slight rise in the intensity of the band was observed, which was restored to control values after insulin treatment (Fig. 4). A significant increase in the 30-kDa band was found in diabetic rats at 14 and 22 days (Fig. 4) and, in the latter, insulin treatment partly restored the levels, which still remained above those of controls. No changes in the 24-kDa band (IGFBP-4) were found at 8 and 14 days, but a slight increase in the amount of this binding protein was observed in diabetic kidneys at 22 days.
days, an increase that was reversed by insulin treatment (Fig. 4).

To differentiate both IGFBPs of the 30-kDa band, Western immunoblots specific for IGFBP-1 and -2 were used. The results depicted in Fig. 5 show an increase in IGFBP-1 in diabetic rats at 14 days and of IGFBP-1 and -2 at 22 days. These results indicate that the diabetes-induced alterations in the renal IGFBP concentration depend on the stage of development of the animal.

Kidney IGFBP-1, -2, and -3 gene expression exhibited considerable variability from animal to animal within the same group: control, diabetic, and insulin-treated rats. No significant differences in kidney mRNA expression levels of IGFBP-1 and -3 were found between control and diabetic rats at 8 and 14 days (Fig. 6) and at 22 days (Fig. 7). Fourteen-day-old diabetic rats exhibited a 50% decrease in IGFBP-2 mRNA transcript levels, which were restored by insulin treatment (Fig. 7). As for IGF-I and -II, a representative experiment on IGFBPs is depicted in Fig. 7, and the results were confirmed by densitometric analysis of three different experiments (9 samples/group) (Figs. 6 and 7). As proof of gel loading normalization, the 18S band is shown below the IGF bands.

Fig. 4. Concentration of IGF binding proteins (IGFBPs) in neonatal rat kidney. Left: in kidney homogenates from control (C) and diabetic (D) rats at 8 and 14 days of life and control, diabetic, and insulin-treated rats (I) at 22 days of life, 45 (IGFBP-3), 30 (IGFBP-1 and -2), and 24-kDa (IGFBP-4) IGFBPs as determined by Western ligand blot (WLB). Right: densitometric values of WLB bands averaged from 2 different experiments (6–10 samples/group) as percentage of control values. ●, $P < 0.05$ relative to control group. ▲, $P < 0.05$ relative to diabetic group.

Fig. 5. Levels of IGFBP-1 and -2 in neonatal rat kidney. Left: representative Western immunoblot (WIB) experiments specific for IGFBP-1 and -2 in kidney homogenates from control (C) and diabetic rats (D) at 14 and 22 days of life. Right: densitometric values of WIB bands averaged from 2 different experiments (8–10 samples/group) as percentage of control values. ●, $P < 0.05$ relative to control group.
DISCUSSION

Because of the important role of the GH/IGF/IGFBP system in renal hemodynamic function (14, 15), the regulation of the system in the kidney is of considerable interest. Both IGF-I and -II have been found in cortex and medulla of rat kidney (14, 15), and mRNA of IGF-II and its receptor have been measured in the rat nephron (13). The renal IGF system is regulated by the nutritional status; i.e., short-term fasting decreases renal IGF-I mRNA and raises renal IGFBP-1 levels in GH-deficient rats, suggesting that these changes are GH independent (26). Similar changes in the renal IGF system have been reported in insulin-deficient diabetes (14), a condition of cellular undernutrition due to the lack of insulin. Our previous results showed that neonatal diabetes induced a decrease in serum concentration and liver mRNA expression of IGF-I and an increase in serum levels and hepatic mRNA expression of IGFBP-1 (21, 36, 37). In the present study, untreated diabetic neonatal rats exhibited an elevated kidney weight-to-body weight ratio, decreased IGF-I and increased IGF-II peptide concentration, and decreased renal mRNA levels of both transcripts, together with an increase in the 30-kDa IGFBPs in kidney. Treatment with insulin for a period of 4 days recovered most of the above parameters.

Kidney enlargement is a well-known feature of early type I (insulin-dependent) diabetes in adult humans and in STZ-induced diabetes in postpubertal rats (34, 39, 40, 42). Because diabetes-induced kidney hypertrophy is accompanied by a reduction in body weight of adult rats, increased kidney weight-to-body weight ratios have been reported in diabetic adult animals (16, 25, 27, 29). However, no increment in the size and weight of kidneys was found in prepubertal animals submitted to diabetes by STZ administration (32). In agreement with the above, the present study showed no differences in the weight of kidneys between diabetic and control neonatal rats, but a significant increase in kidney weight-to-body weight ratio was found in the neonatal diabetic rats that was not reversed by insulin treatment for 4 days. Because no sustained increase was found in the organ weight-to-body weight ratios for liver, spleen, pancreas, and brain in the diabetic group despite the reduced body weight, the rise of kidney weight-to-body weight ratio in diabetic neonates indicates a diabetes-induced maintenance of kidney weight compared with the rest of the organs. However, changes in the hydration balance of the diabetic animal might also be involved in the increase in the kidney weight-to-body weight ratio. IGFs, among other putative growth factors, could be involved in the higher growth ratio of this organ in a neonatal diabetic situation, as in adults (14, 32). Kidney enlargement in conditions of insulin-deficient diabetes in adults is mediated by a GH-dependent increase of IGF-I synthesis (15, 23). However, kidney growth during development appears to be modestly pituitary dependent (19), and reduced plasma GH has been reported in adult (20) and neonatal (21, 36) diabetic rats. Therefore, the results suggest that the diabetes-induced increase in kidney growth-to-body weight ratio during the neonatal period is not mediated by GH.

Diabetic renal hypertrophy in adult rats is preceded by a rise in extractable IGF-I in the kidney (16, 18), suggesting a trophic effect of IGF-I on kidney tissue. Insulin treatment of these rats prevents both the diabetes-induced IGF-I increase as well as kidney hypertrophy (16, 18). However, in immature stages of the rat's development, the most abundant IGF in the liver
is IGF-II, and IGF-I becomes the predominant IGF only after the third week of life (11, 37). Furthermore, in prepubertal diabetic rats, the extractable IGF-I levels were much lower than in control rats (32). In agreement with the above results, reduced IGF-I levels were found in kidney of diabetic neonates at 22 days, indicating that IGF-I response to diabetes in developing rats is similar to that of prepubertal rats and that IGF-I is probably not involved in the increased growth ratio of kidney induced by diabetes. However, a relevant increase in IGF-II was found in kidney of diabetic neonates at 8 and 22 days. Diabetes-induced changes in IGF-II have been reported in serum of adult diabetic rats (4) and serum and liver of neonatal diabetic rats (21, 36). To our knowledge, no data concerning kidney IGF-II in diabetes have as yet been reported, despite the fact that in vitro assay measurements of IGF-II levels after extraction of the peptide from fetal kidney have confirmed the high renal levels, which are only surpassed by the IGF-II concentration in the pituitary (28). Both IGFs have been shown to be growth factors for kidney cells (22, 24), and overexpression of IGF-II in neonatal transgenic mice had no significant effect on body size or growth of most organs but significantly increased kidney weight (43). Therefore, because GH does not play a relevant role in IGF regulation during immature stages (19, 21) and IGF-I and insulin are reduced in neonatal diabetes, the data suggest that the diabetes-induced increase in the growth ratio of the kidney during the neonatal period could be mediated by renal IGF-II accumulation. Insulin treatment for 4 days completely recovered the IGF-I reduction and IGF-II increase, suggesting that both changes are insulin dependent, and no other factors seem to be involved in the regulation of the IGF in the kidney during the neonatal period.

In the present study, induction of STZ diabetes during the neonatal period led to a significant decrease in IGF-I and -II mRNA expression in kidney. Furthermore, the observed decrease in IGF-I mRNA expression was recovered after a 4-day treatment with insulin, suggesting again the insulin dependence of renal IGF-I gene expression during neonatal development. A similar regulation of serum levels and liver mRNA expression of IGF-I and -II in diabetic neonatal rats has been reported previously (21), indicating that the diabetes-induced decrease in IGF-I and -II mRNA expression in the kidney was not organ specific. It is worth noting that IGF-II mRNA expression in kidney of 22-day-old rats was almost undetectable by RNase protection assay, indicating that the adult pattern for the IGF-I/IGF-II ratio had been reached. In a healthy neonatal kidney, IGF-I and -II peptide concentration resulted mostly from in situ mRNA synthesis, as shown by the good correlation between peptide levels and mRNA expression of IGF-I and -II found in kidney of control rats. This correlation was altered in a diabetic condition most likely by changes in IGF uptake by the kidney, because renal levels of IGF-II were increased, whereas its local mRNA expression was reduced. An elegant study has shown an accumulation of extractable IGF-I in kidneys from diabetic postpubertal rats is associated with a seemingly paradoxical decline in renal IGF-I mRNA (32). This finding suggests that the accumulation of IGF-I in the kidney in early diabetes results from increasing binding or uptake rather than in situ synthesis (32). Similarly, increased renal uptake of IGF-I through local IGFBPs was suggested in the absence of variations in IGF-I receptor mRNA in prepubertal diabetic rat kidney (32), but IGF-I/IGF-II receptor binding in neonatal diabetic rats remains to be determined. Although serum IGF-II levels have been found slightly decreased in diabetic neonatal rats (21, 36), our results indicate that the increase in renal extractable IGF-II levels found in neonatal diabetic rats may have resulted from increased uptake of circulating IGF-II by binding to renal IGF receptors or binding proteins.

IGFBPs play a relevant role in IGF regulation in most tissues, including serum, liver, and kidney. Altered renal synthesis of IGFBPs in insulin-deficient diabetes mellitus has been reported, suggesting a role for these proteins in the diabetes-induced alteration in renal function and morphology (27). Increased serum levels and liver mRNA expression of IGFBP-1 have

Fig. 7. Kidney IGFBP-1, -2, and -3 gene expression in 22-day-old rats. Total RNA was extracted from kidney of control (C), diabetic (D), and insulin-treated 22-day-old rats (I), and IGFBP-1, -2, and -3 transcript abundance was determined by RPA. Top: bands from a representative experiment. 18S ribosomal antisense assayed in the same samples was used for lane loading control, and the results are shown beneath the IGFBP bands. + and −, Riboprobe lanes treated with or without RNases, respectively. Bottom: densitometric results in optical density percentage of control values representing the mean of 3 different experiments (9 samples/group). ◼, P < 0.05 relative to control group. ▲, P < 0.05 relative to diabetic group.
been reported in diabetic neonates (21, 36), and insulin treatment for 4 days fully recovered both parameters to those in control rats (21), supporting the inhibitory role of insulin in liver mRNA expression and circulating IGFBP-1 in stages of immaturity. The present data show that the increase in the renal 30-kDa band observed in 14- and 22-day-old diabetic neonates resulted mostly from a rise in IGFBP-1 at 14 days and IGFBP-1 and -2 at 22 days, indicating that the renal 30-kDa IGFBP response to diabetes, as occurs in other tissues (21, 36), depends on the stage of neonatal development. This reverse correlation between insulin and IGFBP-1 in kidney, which was also observed for IGFBP-2 in the 22-day-old rats, agrees with data of other authors that reported increased levels of IGFBP-1 in renal cortex in diabetes (25, 27). An increase in IGFBP-1 mRNA expression has been reported in the kidney of pre- and postpubertal rats within 24 h of the induction of diabetes by STZ (32), whereas in the present study, no changes in IGFBP-1 mRNA expression were found in kidney of neonatal rats 4 days after the onset of diabetes. Perhaps the mRNA response of IGFBPs to diabetes is a rapid and transient event that can be detected only for short periods after induction of diabetes. In addition, the increase in IGFBP-1 protein observed in 14- and 22-day-old rats, 4 days after STZ administration, might result from a posttranscriptional/posttranslational regulation, or increased kidney uptake of IGFBP-1 from the circulation. The latter would be favored by the increased liver gene expression and serum concentration of IGFBP-1 found in diabetic neonatal rats (21, 36).

The possibility of increased uptake of 30-kDa IGFBPs by the kidney has been previously suggested for IGFBP-2 (17). In that study, elevated IGFBP-2 was believed to be the cause of the increase in the 30-kDa band in kidney of diabetic rats. Because no changes in IGFBP-2 mRNA expression have been observed in the same conditions by other authors (27), one may argue that this IGFBP could have been trapped by binding to membrane integrins (14). In the present study, the elevated IGFBP-2 concentration, together with its reduced mRNA expression in the kidneys of 22-day-old diabetic rats, seems to suggest, as in adult diabetic patients, an increased uptake of circulating IGFBP-2 by the diabetic kidney. Because the 30-kDa IGFBPs show a high affinity for IGF-II (35), the diabetes-induced increase in the 30-kDa IGFBPs in neonatal kidneys could favor the accumulation of renal IGF-II and, consequently, increase the local growth effect, resulting in a higher renal growth rate.

Insulin deficiency seems to regulate IGFBP-3 levels in kidney of 22-day-old neonatal rats, because insulin treatment restored the STZ-induced increase in the 45-kDa band. In adult rats, a decrease in the renal 38- to 47-kDa doublet band, corresponding to the insulin-like growth factor binding subunit of the IGFBP-3, followed a transient increase during the first 2 days after the induction of diabetes, and insulin treatment prevented these changes (17). Perhaps during the weaning stage (22 days of life), a transition period for the rat between the neonatal to adult pattern of IGF regulation, the renal response to insulin deficiency could be somewhat delayed. However, the fact that insulin restores IGFBP-3 levels in the neonatal kidney points out the influence of the metabolic status on the renal IGFBPs.

Diabetic rats have been reported to have normal serum IGFBP-4 and a moderate decrease in its mRNA expression in kidney (9, 27). Our previous data have shown no changes in the serum 24-kDa band (IGFBP-4) of diabetic neonatal rats at the same stages (36). Because kidney mRNA expression of IGFBP-4 could not be measured in this study, the slight increase in the IGFBP-4 concentration in the kidney of diabetic neonates at 22 days could result from altered gene expression, posttranscriptional regulation, and/or increased uptake from circulation as suggested for other IGFBPs. In any case, the physiological role of IGFBP-4 in diabetic nephropathy, either neonatal or adult, remains to be determined.

Intensive research has been carried out to unravel IGF regulation in diabetic kidney at peripubertal and adult stages, and the number of discrepancies in experimental data from several authors shows the difficulty of this study (14). The first attempt to study this system in diabetic kidney during neonatal stages of development is described in this study. Some responses different from those of mature kidney are reported herein. The main finding appears to be the diabetes-induced IGF-II accumulation in neonatal kidney, which could be responsible for the elevated growth ratio of the kidney compared with other organs in diabetes during neonatal stages, as IGF-I is responsible for the same effect in mature animals.

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