Kidney IGF-I mRNA in initial renal hypertrophy in experimental diabetes in rats

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Summary. It has recently been demonstrated that immunoassayable kidney insulin-like growth factor I concentration increases 24–48 h after induction of diabetes, preceding the initial renal hypertrophy. To elucidate whether this increase is due to increased local production we studied rat kidney insulin-like growth factor I gene expression during the first four days after induction of streptozotocin diabetes. Eighteen hours after injection with streptozotocin the diabetic animals were divided into two groups, one of which was treated with insulin, and daily for four days animals from each group were taken out for investigation. After four days the wet kidney weight had increased from baseline by 20% (from 687 ± 23 to 827 ± 6 mg (mean $\pm$ SEM), $p < 0.01$) in the untreated diabetic group, while no significant increase occurred in the insulin-treated group (687 ± 23 vs 732 ± 21 mg, NS). Kidney insulin-like growth factor I increased rapidly from baseline, the rise amounting to 52% after 48 h (from 271 ± 11 to 411 ± 32 ng/g, $p < 0.01$) with a decline to control level on day four in the untreated diabetic group. Kidney insulin-like growth factor I remained unchanged in the insulin-treated diabetic group. Insulin-like growth factor I mRNA was measured by solution-hybridization assay. No differences were found in kidney insulin-like growth factor I mRNA between the two diabetic groups over the study period, while in liver, insulin-like growth factor I mRNA tended to be lower on day four in diabetic rats when compared to insulin-treated rats ($p = 0.07$). These results show that the increase in kidney insulin-like growth factor I during initial renal hypertrophy in experimental diabetes is not associated with an elevated level of kidney insulin-like growth factor I mRNA and suggest that other, possibly translational, mechanisms are responsible for insulin-like growth factor accumulation in the kidney.

Key words: Renal hypertrophy, insulin-like growth factor I, insulin-like growth factor I mRNA, streptozotocin diabetes.

Renal hypertrophy and hyperfiltration are early manifestations of human and experimental diabetes [1–3] which may contribute to the later development of diabetic nephropathy [4]. Induction of experimental diabetes causes a rapid increase in renal size accompanied by increases in total kidney RNA and protein demonstrable 36 h after the onset of glycosuria, whereas DNA content remains unchanged for at least the first eight days [2]. This diabetic renal hypertrophy is preceded by a rise in extractable renal tissue concentration of insulin-like growth factor I (IGF-I), which reaches a peak 48 h after induction of diabetes [3, 5].

It is not known whether this increase in kidney IGF-I concentration is due to increased uptake from the blood or to local production of IGF-I. Traditionally, the liver has been considered to be the main site of IGF-I synthesis mainly under the control of growth hormone [6]. However, the increase in kidney IGF-I after induction of diabetes is accompanied by little or no change in circulating levels of IGF-I [3, 5]. RIA of acetic acid tissue extracts

has demonstrated the presence of IGF-I in many tissues including the kidney and liver [7] and recently the gene expression for IGF-I, measured as tissue content of mRNA for IGF-I (IGF-I mRNA), has been demonstrated in many tissues including the kidney [8, 9].

Renal hypertrophy following growth hormone (GH) treatment of hypophysectomized rats and compensatory renal growth subsequent to unilateral nephrectomy are both accompanied by increased levels of extractable [3, 5, 7, 10–12] and immunostainable kidney IGF-I [11, 12]. GH-treatment induces increased renal IGF-I mRNA expression, whereas unilateral nephrectomy is followed by unchanged, steady-state IGF-I mRNA levels [12]. These results suggest that IGF-I may act as a renotropic agent through both transcriptional and translational mechanisms.

In this study we investigated the mechanism responsible for kidney IGF-I accumulation in experimental diabetes by measurement of kidney IGF-I mRNA during the initial renal growth following induction of diabetes.

Materials and methods

Animals

Male Wistar rats (Møllegaards Avlslab., Eiby, Denmark) with a mean body weight of 195 g (range 185–204 g) were studied. Rats were housed three per cage in a room with 12:12 h (06.00–18.00 hours) artificial light cycle, temperature 21 ± 2°C and humidity 55 ± 2%. The animals had free access to standard rat chow (Altromin, Lage, FRG) and tap water throughout the experiment. The animals were randomised into three groups matched for body weight: (1) control animals ($n = 12$); (2) diabetic animals, no insulin treatment ($n = 24$) and (3) diabetic animals, insulin-treated ($n = 24$). Diabetes was induced on day 0 by i. v. injection of streptozotocin (STZ) (55 mg/kg body weight) in acidic 0.154 mol/l NaCl (pH 4.0) following 12 h of food deprivation. Eighteen hours after administration of STZ, and daily thereafter, the animals were weighed, urinalysis was performed for glucose and ketones using Neostix 4 (Ames Limited, Stoke Poges, Slough, UK) and tail-vein blood glucose determined by Haemoglucotest 1–44 and Reflolux II reflectance meter (Boehringer-Mannheim, Mannheim, FRG). Insulin treatment with a very long-acting, heat-treated Ultralente Insulin (Novo, Bagsvaerd, Denmark) was initiated 18 h after administration of STZ when all animals had blood glucose levels above 15 mmol/l. Insulin was given in an initial dose of 4–6 U, followed by 1–2 U daily thereafter depending on blood glucose values.

Each day from day 1 to 4, six animals from each of the two diabetic groups were studied in addition to six control animals on days 0 and 4. Under sodium barbital anaesthesia (50 mg/kg) blood was drawn from the retro-orbital venous plexus and serum stored at − 20°C for later determination of IGF-I. Both kidneys were removed and rapidly cleaned. The left kidneys were immediately frozen in liquid nitrogen for later analysis of IGF-I mRNA and the right weighed, frozen and subsequently used for IGF-I determination by acetic acid extraction. In addition, two liver pieces were removed and immediately frozen for later IGF-I mRNA and IGF-I analysis.

Preparation of total nucleic acid

Total nucleic acid (TNA) was prepared according to Durnam and Palmiter [13]. In short, the frozen tissues were homogenized in a sodium dodecyl sulphate (SDS)-containing buffer with a polytron PT 1020 350D (Kinematica GmbH, Luzern, Switzerland) for 8 s. The samples were digested with proteinase K and extracted with phenol and chloroform. The TNA was precipitated by addition of ethanol, and measured by spectrophotometry at 260 nm, while the DNA-content was measured by fluorimetry [14].

Solution-hybridization of IGF-I mRNA

Levels of IGF-I mRNA were analysed using a (^{35}S)UTP-labelled RNA probe. The probe was complementary to 153 basepairs in exon 3 (by analogy to the human gene) of the mouse IGF-I gene as described previously [8]. This sequence has 92% homology with the corresponding rat sequence, and it detects the different forms of IGF-I mRNA which have recently been described in rat tissues [15]. The RNA probe, prepared according to the method of Melton [16], was hybridized to TNA samples at 70°C for 16 h as described by Mathews et al. [8]. Hybridization was performed in 40 μl of 0.6 mol/l NaCl, 20 mmol/l Tris-HCl (pH 7.5), 4 mmol/l EDTA, 0.1% (weight/volume) SDS, 7.5 mmol/l dithiothreitol (DTT), 25% (v/v) formamide and 10,000 cpm probe/incubation (specific activity; approximately 1.2×10^{15} cpm/g probe). The samples were exposed to RNAses and the hybrids precipitated with 100 μl of 6 mol/l trichloroacetic acid, collected on glass microfibre filters and analysed in a liquid scintillation counter (LKB, 1217 Rackbeta, Turku, Finland). The radioactivity of the TNA samples was compared with that of a standard curve constructed from incubations with a rat liver TNA sample with a known amount of IGF-I mRNA. The level of mRNA in the TNA standard sample was originally determined using known amounts of in vitro synthesized RNA complementary to the probe. A standard curve was included in each assay and the samples were analysed in triplicate. The intra-assay and the inter-assay coefficient of variation of the method was 22% and 20% respectively, when a sample with a very low concentration of IGF-I mRNA (13.1 pmol/l IGF-I mRNA) was analysed six times.

IGF-I determination

Tissue extraction of renal and hepatic IGF-I was performed in 1 mol/l acetic acid as described by D'Ercole [7]. After extraction and lyophilization the samples were redissolved in 40 mmol/l phosphate buffer (pH 8.0) and stored at − 20 °C until analysis [3, 5]. Serum was extracted in acetic acid-methanol [3, 5]. IGF-I was determined by RIA using IGF-I antibody UB 286 (raised by L. E. Underwood and J. J. van Wyk, Paediatric Endocrinology, University of North Carolina, Chapel Hill, NC, USA) donated by the US National Hormones and Pituitary Program. A full amino-acid sequence analogue (Amgen Biologicals, Thousand Oaks, Calif., USA) was used for standards and iodination [3, 5].

Chemicals

SDS and DTT were obtained from Sigma Chemical Co., (St. Louis, Mo., USA) and Proteinase K from Merck, (Darmstadt, FRG). Phenol (liquified) was obtained from Fisher Scientific, (Fair Lawn, NJ, USA). (^{35}S)UTP and IGF-I were obtained from Amersham International plc., (Amersham, Buckinghamshire, UK). Chemicals for probe synthesis were obtained from Promega Inc., (Madison, Wi., USA) and RNase A, RNase T1 and herring sperm DNA were obtained from Boehringer. Insta-Gel was obtained from Packard, (Groningen, The Netherlands) and formamide was from IBI, (New Haven, Ct., USA).

Statistical analysis

Differences between groups were analysed by one-way analysis of variance (ANOVA) in combination with the Bonferroni test for multiple comparisons and unpaired Student's t-test. Significant differences between untreated and insulin-treated animals are shown in the figures, while the significances of changes within groups from day 0 level are given in the text. Results are expressed as mean ± SEM.

Results

Metabolic parameters and body weight (Fig. 1)

All animals given STZ developed diabetes with blood glucose concentrations above 15 mmol/l after 18 h (Fig. 1 a). Blood glucose in the untreated diabetic animals stabilized around 25 mmol/l after 24 h and remained at that level for the duration of the study, whilst in the insulin-treated group blood glucose fell to around 6 mmol/l 6 h after the first injection and remained stable thereafter at levels comparable to non-diabetic control animals (Fig. 1 a). All untreated animals had glycosuria of > 111 mmol/l and none of the animals exhibited ketonuria at any time during the study.

Body weight changes are shown in Figure 1 b. Untreated diabetic animals had a weight loss of 4% on day 2 from initial level ($p < 0.01$), returning towards initial levels
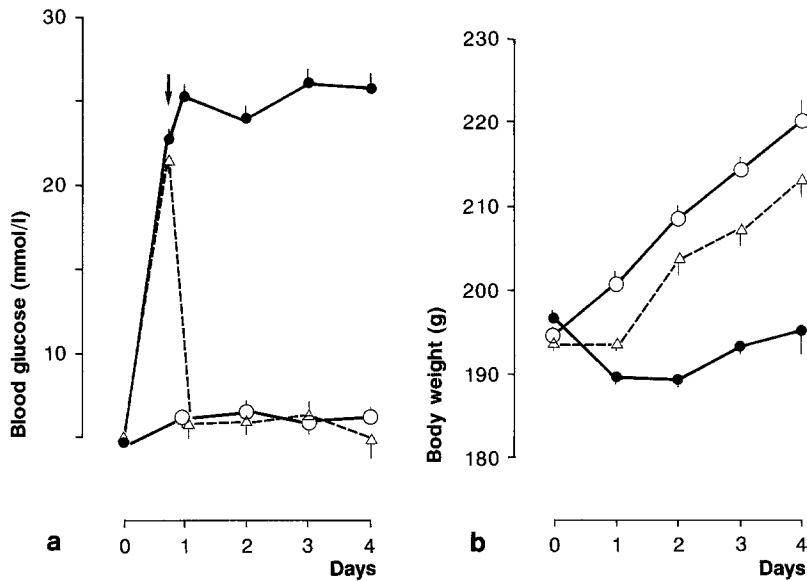
Fig. 1 a, b. Blood glucose (a) and body weight (b) during the four experimental days in control animals (O—O), untreated diabetic (●—●) and insulin-treated diabetic animals (△---△). Insulin treatment with a very long-acting, heat-treated Ultralente Insulin was initiated 18 h after administration of streptozotocin, as indicated by the arrow. Values are mean ± SEM. Blood glucose and body weight values include all animals on day 0 and from day 1 to 4 each point represents the remaining animals i.e. $n = 24, 18, 12, 6$ in each of the diabetic groups. For control animals each point represents $n = 6$

on days 3 and 4. Insulin-treated animals did not lose weight, and had, after the initiation of insulin treatment, a weight gain parallel to that seen in non-diabetic control animals (Fig. 1 b).

Kidney growth (Fig. 2)

Kidney weight in the untreated group increased rapidly after induction of diabetes, significant differences from baseline being seen by day 3 ($p < 0.02$) (Fig. 2) and after 4 days wet kidney weight had increased from baseline by 20% (from 687 ± 23 to 827 ± 6 mg) ($p < 0.01$). Kidney weight in the untreated group was significantly greater ($p = 0.01$) than in the insulin-treated group after 24 h and throughout the observation period. The kidney weight of the control animals did not change significantly from day 0 to 4, being identical to kidney weight of insulin-treated diabetic animals (Fig. 2).

Kidney IGF-I levels by RIA (Fig. 3 a)

The kidney IGF-I concentration (ng/g kidney tissue) increased rapidly in the untreated diabetic group reaching a peak 2 days after induction of diabetes, with an increase of 52% from the initial level (from 271 ± 11 to 411 ± 32 ng/g, $p < 0.01$). The kidney IGF-I concentration thereafter declined to control values on day 4. There was no change in renal IGF-I concentration in either insulin-treated diabetic or control animals during the study period. Kidney IGF-I levels were significantly higher in the untreated group on day 2 and 3 when compared to their insulin-treated counterpart (Fig. 3 a).

Kidney IGF-I mRNA levels (Fig. 3 b)

IGF-I mRNA expressed as amount of IGF-I mRNA/DNA ($a(10^{-18})$mol/µg), was quantified using solution-hybridization assay as described in Materials and methods. Figure 3 b shows that IGF-I mRNA levels were

unchanged and similar in both diabetic groups and in non-diabetic controls during the first 4 days of diabetes.

Hepatic IGF-I mRNA, extractable hepatic IGF-I and serum IGF-I levels (Table 1)

No significant differences were found in liver IGF-I mRNA or IGF-I levels between the two diabetic groups during the study period, although liver IGF-I mRNA tended to be lower at day 4 in untreated diabetic rats when compared to the insulin-treated group ($p = 0.07$). Serum
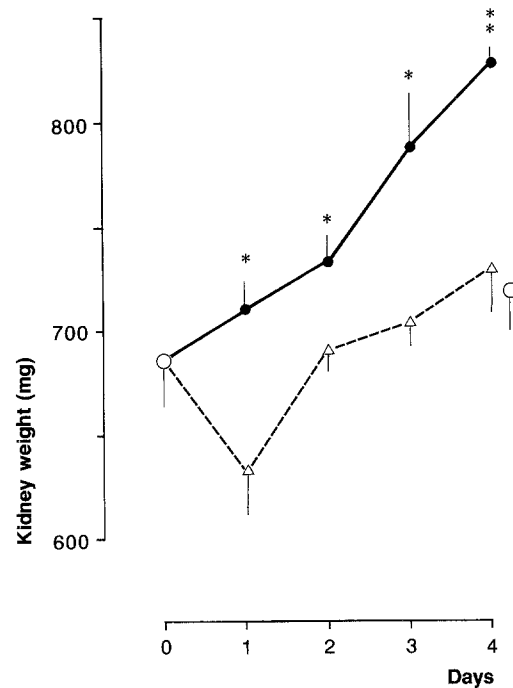

Fig. 2. Changes in kidney weight in streptozotocin-diabetic rats, untreated (●—●) and insulin-treated (△---△), over four days and in non-diabetic control rats day 0 and 4 (O). *$p < 0.05$, **$p < 0.01$ between untreated and insulin-treated diabetic animals. Values are given as mean ± SEM. Every point represents 6 different animals
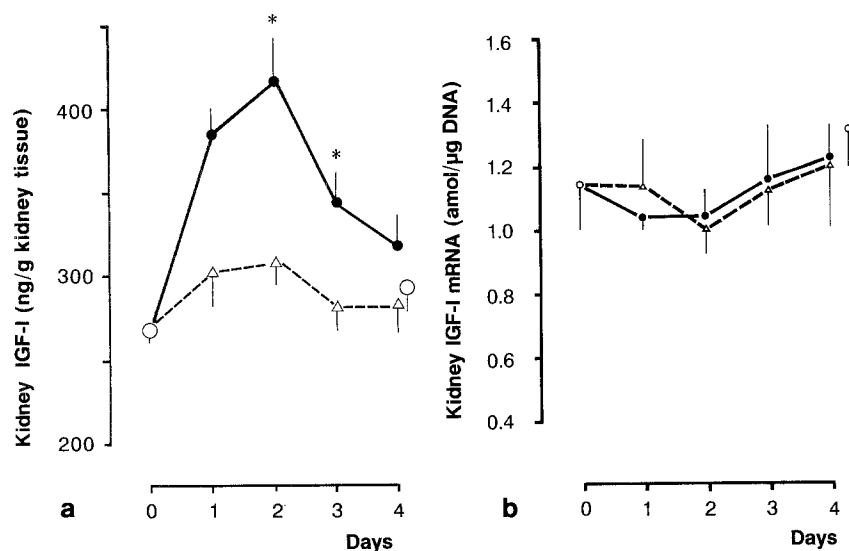
Fig. 3a, b. Kidney insulin-like growth factor I (IGF-I) (**a**) and kidney IGF-I mRNA (**b**) in control (0), untreated diabetic (●—●) and insulin-treated diabetic animals (△---△) during the four experimental days. *$p < 0.05$ between untreated and insulin-treated diabetic animals. Values are mean ± SEM, $n = 6$ for kidney IGF-I values and $n = 5$ for kidney IGF-I mRNA

IGF-I was decreased in untreated diabetic animals on day 2 when compared to their insulin-treated counterpart ($p < 0.01$), while no difference was found on day 4 (Table 1).

Discussion

The demonstration of IGF-I mRNA in kidney in this and other studies [8, 9, 12, 17, 18] suggests that the kidney is capable of synthesizing IGF-I. The level of kidney IGF-I mRNA was approximately 100-fold lower than that in liver. Local production of immunoreactive IGF-I has been demonstrated in several types of cells including kidney mesangial cells [19, 20]. In this study we found unchanged levels of IGF-I mRNA in diabetic kidneys although immunoreactive IGF-I concentration was increased. Two recent reports have shown decreased kidney IGF-I mRNA levels in experimental diabetes of four [17] and seven days duration [18]. The different results may be due to differences in severity of the diabetic state. Although the degree of renal hypertrophy is linearly dependent on blood glucose concentration, a too severe metabolic derangement, as indicated by extreme hyperglycaemia or ketonuria, diminishes or even abolishes the kidney growth [21–23]. We found that IGF-I mRNA in liver and IGF-I in serum

tended to be lower in untreated diabetic rats compared to insulin-treated rats. In rats with a more severe metabolic upset than in this present study IGF-I mRNA in liver [17, 18, 24] and IGF-I in serum [18, 24] were reduced. Serum IGF-I originates mainly from the liver [25] and these results suggest that the lowered IGF-I mRNA in liver is accompanied by a decrease in synthesis of circulating IGF-I. The finding of unaffected or decreased IGF-I mRNA levels in the diabetic kidney suggests that the local production of IGF-I is unchanged or decreased in diabetic kidneys undergoing initial renal hypertrophy.

In two other situations with rapid initial renal growth: administration of GH to hypophysectomized rats and unilateral nephrectomy, similar rises in extractable and immunostainable renal IGF-I concentration and kidney weights are seen [3, 5, 7, 10–12]. Consistent with a GH-mediated induction of IGF-I synthesis, systemic GH-treatment enhances kidney levels of IGF-I mRNA, immunostainable IGF-I and kidney size [12]. Renal growth following unilateral nephrectomy has been reported to be accompanied by a "relative" increase in renal IGF-I mRNA using "dot-blot" analysis [26], however, when using solution-hybridization assay with higher specificity and sensitivity [27] no changes in steady-state IGF-I mRNA level in kidneys from nephrectomized rats were seen [12].

Table 1. Hepatic insulin-like growth factor I (IGF-I) mRNA, extractable hepatic IGF-I and serum IGF-I in untreated and insulin-treated rats on day 2 and day 4 and in non-diabetic control rats on day 4. Values are given as mean ± SEM. Numbers in parentheses indicate the number of samples analysed

	IGF-I mRNA ($a(10^{-18})$mol/µg DNA)		IGF-I (ng/g liver)		Serum IGF-I (µg/l)	
	Day 2	Day 4	Day 2	Day 4	Day 2	Day 4
Untreated diabetic rats	117 ± 24 (6)	98 ± 22 (5)	47 ± 4 (6)	57 ± 6 (6)	733 ± 80[a] (6)	941 ± 40 (6)
Insulin-treated diabetic rats	171 ± 22 (6)	184 ± 37 (5)	54 ± 5 (6)	54 ± 7 (6)	1115 ± 88 (6)	882 ± 33 (6)
Non-diabetic control rats	–	142 ± 18 (6)	–	70 ± 13 (6)	–	1036 ± 70 (6)

[a] $p < 0.02$ between untreated and insulin-treated diabetic rats on day 2

The solution-hybridization assay used in the present study is sensitive enough to detect small differences in IGF-I mRNA [13]. The unaltered level of IGF-I mRNA in renal hypertrophy in experimental diabetes seems to indicate that the local production at transcriptional level is unchanged. The major regulation of renal IGF-I production in GH-induced renal hypertrophy has been found to be pre-translatory [12]. However, it cannot be excluded that the IGF-I accumulation in the diabetic kidney is due to enhanced translatory regulation, as it has been suggested for the IGF-I accumulation in post-nephrectomy renal growth [12].

Alternatively, other mechanisms may account for the increase in immunoreactive IGF-I in diabetic kidneys. Circulating or locally produced IGF-I could be trapped more efficiently, for example via enhanced receptor-mediated binding to cells or via an altered pattern of IGF-I binding protein expression. Finally, sequestration of IGF-I within tubules and collecting ducts or decreased breakdown of the peptide could be responsible for the IGF-I accumulation in the diabetic kidney.

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