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Rapid communication

Transient increase in renal insulin-like growth factor binding proteins during initial kidney hypertrophy in experimental diabetes in rats

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Summary. The insulin-like growth factors, insulin-like growth factor I and insulin-like growth factor II are bound to six distinct classes of insulin-like growth factor binding proteins (IGFBPs) in the circulation and in extracellular fluids. Diabetic renal hypertrophy is preceded by a transient increase in kidney insulin-like growth factor I suggestive of a renotropic function for insulin-like growth factor I. In order to examine a possible involvement of IGFBPs in initial diabetic kidney growth and in kidney insulin-like growth factor I accumulation, we studied rat kidney IGFBPs by ligand blotting during the first 4 days after induction of diabetes. Six distinct bands were identified in kidney and liver tissue with apparent molecular weight values of 38–47 (doublet), 34, 30, 24 and 20 kDa. The 38–47 kDa doublet band probably corresponds to the insulin-like growth factor binding subunit of IGFBP-3, the 24 kDa band to IGFBP-4 and the 30 kDa band to IGFBP-1 and/or IGFBP-2, as these IGFBPs in rats have similar molecular weight. In untreated diabetic rats a transient increase in the kidney 30 kDa band was demonstrable 24 h after induction of diabetes with a maximal rise (two-fold) after 48 h, followed by a decrease to baseline values after 4 days. In untreated diabetic rats the 38–47 kDa doublet band also increased (two-fold) in kidney during the first 2 days after induction of diabetes, followed by a subsequent decrease. Insulin-treatment prevented both the increase in the 30 kDa and in the 38–47 kDa bands. Kidney weight in untreated diabetic rats increased by 26% after 4 days. In conclusion, the present study shows a transient increase in the 30 kDa and the 38–47 kDa IGFBP species in hypertrophying diabetic kidneys, contemporarily with the previously described transient increase in extractable kidney insulin-like growth factor I content. These findings support the concept that IGFBPs may be involved in the action of insulin-like growth factor I and possibly in the diabetic kidney insulin-like growth factor I accumulation.

Key words: Kidney, hypertrophy, insulin-like growth factors, insulin-like growth factor binding proteins, streptozotocin, diabetes, rat.

The insulin-like growth factors, IGF-I and IGF-II are bound to specific IGF binding proteins (IGFBPs) in the circulation and in extracellular fluids. Six different IGFBPs have been characterized and designated IGFBP-1 to -6 [1]. Human (h) IGFBP-1 and hIGFBP-2 are 28 kDa and 31 kDa proteins, while rat (r) IGFBP-1 has a molecular weight similar to rIGFBP-2 (29 kDa) [1]. IGFBP-1 and -2 are primarily regulated by insulin [1] and largely growth hormone (GH)-independent [1], while IGFBP-3 is GH-dependent and the predominant carrier of IGFs in the circulation [1]. IGFBP-3 is composed of two parts forming a 150 kDa complex: an IGF binding, acid-stable glycoprotein which on gel electrophoresis appears as a major and minor band, corresponding to molecular weight 47 and 38 kDa [1] and an acid-labile, non-IGF-binding subunit with a molecular weight of 84–86 kDa. The IGFBP-4, -5 and -6 have only recently been characterized and little is known about their tissue distribution and function [1].

IGFBPs may play an important role as local modulators of IGF actions. Such mechanisms could especially be operative for IGFBP-1 and -2, since these binding proteins contain an Arg-Gly-Asp tripeptide, which implies that they may interact with cell surfaces [2]. Induction of streptozotocin (STZ)-diabetes mellitus causes a dramatic increase in hepatic mRNA IGFBP-1 and -2 levels, and circulating IGFBP-1 levels show a four-fold increase 2 to 3 days after induction of diabetes [3–5]. A renotropic function for IGF-I has been proposed in diabetic kidney growth [6]. The rapid renal hypertrophy becomes significant 48–72 h following induction of diabetes and is preceded by a transient rise in renal IGF-I concentration, reaching a peak after 24–48 h and returning to basal levels after about 4 days [6].

In order to assess a possible involvement of IGFBPs in the kidney IGF-I accumulation and initial diabetic renal growth, we measured rat kidney IGFBPs by ligand blotting during the first 4 days after induction of STZ-diabetes.

Materials and methods

Animals

Male Wistar rats (Møllegaards Avlslab., Eiby, Denmark) with a mean body weight of 213 g (7–8 weeks of age) 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 randomized into three groups matched for body weight: (1) control animals ($n = 6$); (2) diabetic animals, no insulin treatment ($n = 24$) and (3) diabetic animals, insulin-treated ($n = 24$). Diabetes was induced at day 0 by i.v. injection of 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 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-Nordisk, Bagsværd, Denmark) was initiated 18 h after administration of STZ when all animals had blood glucose levels above 20 mmol/l. Insulin was given in an initial dose of 4–6 IU, followed by 1–3 IU daily thereafter depending on blood glucose values. On days 1, 2, 3 and 4, six animals from each of the two diabetic groups were studied in addition to six control animals on day 0. Under sodium barbital anaesthesia (50 mg/kg body weight) blood was drawn from the retro-orbital venous plexus and serum stored at −80 °C. The animals were rapidly dissected to obtain the kidneys and liver. The tissues were rapidly blotted, weighed and immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Tissue extraction

Approximately 20 mg of thawed kidney or liver tissue was placed in 1.5 ml polypropylene tubes and weighed. The tissue was homogenized for 2 min in 0.5 ml of 20 mmol/l Tris, 2 % Triton X-100 buffer (pH 7.4) using a micropestel (RPI catalog number 9922, Research Products International, Mount Prospect, Ill., USA). After adding 0.13 ml of Laemmli buffer [7], each tube was boiled for 5 min and incubated overnight at 4 °C. Aliquots of extracts were stored at −70 °C. Protein content of the extracts was measured using a protein assay (Pierce Rockford, Cat. no. 23225, Rockford, Ill., USA) with bovine serum albumin as standard.

SDS-PAGE and ligand blot analysis of IGFBPs

SDS-PAGE and ligand blot analysis was performed according to the method of Hossenlopp et al. [7]. Three to four animals from each group on day 0, 1, 2, 3 and 4 were randomly selected for IGFBP analysis. Thawed extracts were boiled for 1 min and centrifuged for 1 min at 13,000 rev/min. An aliquot of the supernatant equivalent to 200 µg of protein or in a subset of experiments 2 µl of serum, was subjected to SDS-PAGE (10 % polyacrylamide) under non-reducing conditions. The electrophoresed proteins were transferred by electroelution onto nitrocellulose paper (Schleicher & Schuell, Munich, FRG) and the membranes were incubated overnight at 4 °C with approximately 500,000 cpm ^{125}I-IGF-II (specific activity 2000 Ci/mmol, Amersham International, Amersham, Bucks, UK) in 10 ml 10 mmol/l Tris-HCl buffer (TBS) containing 1 % bovine serum albumin and 0.1 % Tween (pH 7.4). Membranes were washed with TBS and after drying overnight, the nitrocellulose sheets were autoradiographed with Kodak X-AR film and exposed to Du Pont-New England Nuclear enhancing screens at −70 °C for 3–7 days. Specificity of the IGFBP bands was ensured by competitive co-incubation of ^{125}I-IGF-II with unlabeled IGF-II (1 µg/ml) kindly donated by Lilly Research Lab. (Indianapolis, Ind., USA) or Kabi Peptides (Stockholm, Sweden).

Densitometry

Autoradiographs of ligand blots were scanned using a laser densitometer (Ultro Scan XL, LKB, Bromma, Sweden). The relative densities of the bands were measured as arbitrary absorbance units per milimeter.

Statistical analysis

Differences between groups were analysed using unpaired Student's t-test. When more than two groups were compared, one-way analysis of variance with the Bonferroni test for multiple comparisons and unpaired t-test was used. Results are given as mean values ± SEM.

Results

Metabolic parameters (Table 1) and body weight

All animals given STZ developed hyperglycaemia within 18 h with blood glucose concentrations above 20 mmol/l. Blood glucose concentrations in untreated diabetic animals stabilized at approximately 27 mmol/l after 24 h and remained at that level for the duration of the study. In insulin-treated animals blood glucose decreased to around

Table 1. Blood glucose and kidney weight in non-diabetic control rats on day 0 and in untreated and insulin-treated diabetic rats during the 4 days studied

Blood glucose (mmol/l)	Day 0	Day 1	Day 2	Day 3	Day 4
Untreated diabetic rats		27.3 ± 1.0	27.9 ± 0.7	29.0 ± 0.7	27.7 ± 1.0
Insulin-treated diabetic rats		5.1 ± 0.6	4.2 ± 0.6	4.0 ± 0.5	4.1 ± 0.5
Non-diabetic control rats	5.1 ± 0.3				
Kidney weight (mg)	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**
Untreated diabetic rats		772 ± 23[a]	810 ± 21[b]	812 ± 18[b]	873 ± 36[b]
Insulin-treated diabetic rats		685 ± 19	710 ± 17	715 ± 28	693 ± 25
Non-diabetic control rats	693 ± 20				

[a] $p < 0.05$, [b] $p < 0.01$ untreated rats vs insulin-treated diabetic rats. Values are given as mean ± SEM. $n = 6$

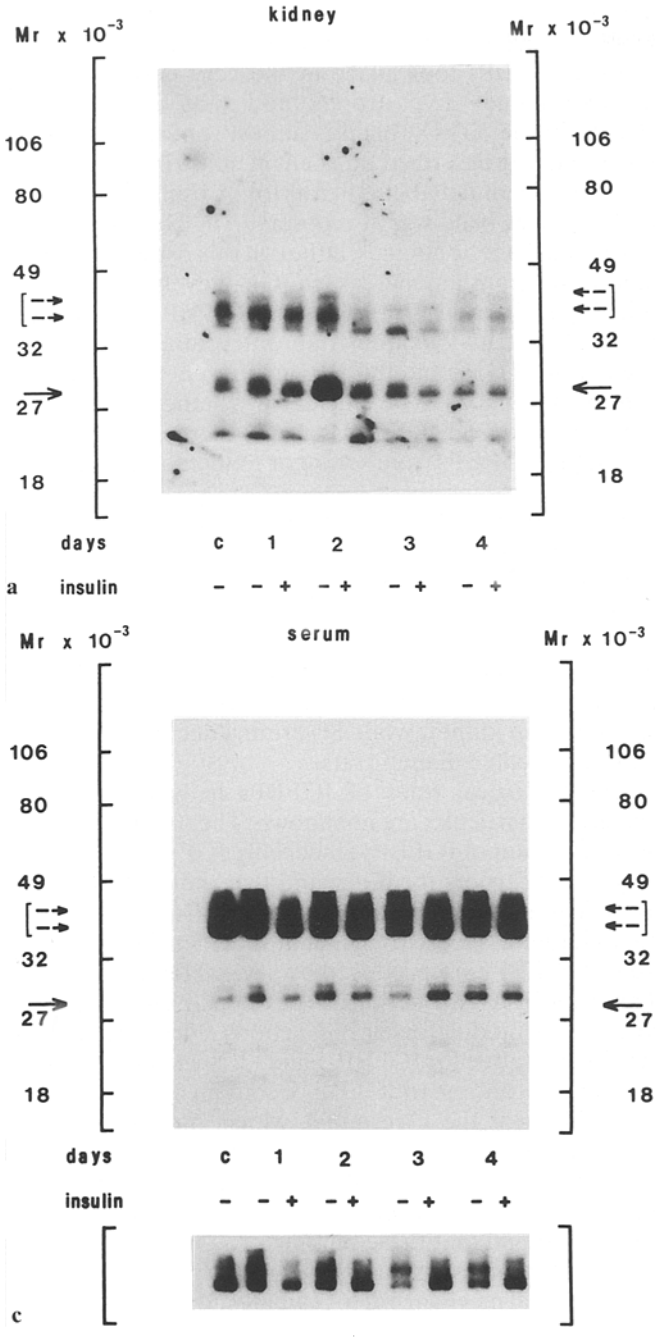

Fig. 1 (a–c). ^{125}I-insulin-like growth factor II ligand blot analysis of kidney **a**, liver **b** and serum **c** insulin-like growth factor binding proteins (IGFBPs) in non-diabetic control (C), untreated diabetic (–) and insulin-treated diabetic (+) animals during 4 days after induction of diabetes. Kidney, liver and serum IGFBPs were separated by SDS-PAGE (10% gel), transferred to nitrocellulose sheets, and ligand blotting was performed as described in Materials and methods. In panel **c**, a briefer exposure (8 h) of the same autoradiograph (upper panel) is also shown to demonstrate the pattern of IGFBP species in the 38–47 kDa region. In all panels [⇉ points to the 38–47 kDa IGFBP species and → points to the main 30 kDa IGFBP species

5 mmol/l 6 h after the first injection and remained stable thereafter with values not different from non-diabetic control animals (Table 1). All untreated diabetic animals had glycosuria greater than 111 mmol/l and none of the animals exhibited ketonuria at any time during the study.

Untreated diabetic animals had a body weight loss of 6% on day 2 ($p < 0.05$), followed by an increase during the study approaching the initial body weight. Insulin-treated diabetic animals did not lose weight, and at day 4, the body weight of insulin-treated diabetic rats was 226 ± 3 g ($n = 6$), significantly different from the body weight in untreated diabetic animals (205 ± 5 g, $n = 6$) ($p < 0.01$).

Kidney weight (Table 1)

A marked increase in kidney weight was observed in untreated diabetic animals, significantly different from insulin-treated diabetic animals as early as 24 h after induction of diabetes ($p < 0.05$) (Table 1). After 4 days of untreated diabetes, kidney weight had increased by 26% when compared to insulin-treated diabetic control rats ($p < 0.01$) (Table 1). Kidney weight in insulin-treated diabetic animals on day 4 was not significantly different from kidney weight in non-diabetic control rats on day 0 (Table 1).

Ligand blots of kidney, liver and serum IGFBPs (Fig. 1)

Autoradiographs of ligand blots of IGFBPs in kidney, liver and serum in untreated and insulin-treated diabetic rats on day 1, 2, 3 and 4 and in non-diabetic control rats on day 0 are shown in Figure 1. Ligand blots of kidney, liver and serum from non-diabetic control rats yielded six bands of IGFBPs with apparent molecular weights of 38–47 kDa (doublet), 34, 30, 24 and 20 kDa. Binding of ^{125}I-IGF-II to these binding species in ligand blotting experiments was specific, since co-incubation of radioligand with unlabelled IGF-II led to complete competitive inhibition of binding (data not shown). In kidney, induction of diabetes resulted in a transient elevation of the 30 kDa band on day 1, with a maximal rise on day 2 (200 ± 61%, $n = 3$) ($p < 0.05$) (Fig. 1 a). The elevation of the 30 kDa

band declined to baseline level on day 3 to 4 (Fig. 1 a). Insulin treatment in diabetic animals abolished the rise in the 30 kDa band. In liver, almost similar changes were seen in the predominant 30 kDa IGFBP species (Fig. 1 b).

In kidney, the 38–47 kDa doublet band also increased on day 1 and 2 in untreated diabetic animals ($200 \pm 52\%$, $n = 3$) ($p < 0.05$) followed by a subsequent decrease on day 3 to 4 in both untreated and insulin-treated diabetic rats (Fig. 1 a). In liver, the 38–47 kDa doublet band also increased in untreated diabetic animals within the first 2 days ($133 \pm 21\%$, $n = 3$) ($p < 0.05$), followed by a subsequent decrease on day 3 and 4 in untreated diabetic animals only (Fig. 1 b).

Changes, largely similar to those described in liver, were detected in the different IGFBP species throughout the study period, when sera from the different groups were subjected to SDS-PAGE (Fig. 1 c).

Discussion

The IGFBPs are a family of structurally related proteins which form simple binary complexes with IGF-I and IGF-II. Six distinct structural classes have been identified, designated IGFBP-1 to IGFBP-6 [1]. The relevance of examining possible changes in the renal IGFBP pattern in diabetic rats was determined by the previous finding that experimental diabetes is associated with a number of changes in circulating and hepatic levels of IGFBPs [3–5, 8]. Furthermore, a transient renal IGF-I accumulation occurs within the first 4 days after induction of experimental diabetes [6].

In the present study we used ^{125}I-IGF-II ligand blotting to characterize IGFBPs by SDS-PAGE analysis. The use of IGFBP antibodies on SDS-PAGE in immunoblotting experiments would provide more specific information of the distinct classes of IGFBPs, but the scarcity of specific antibodies against rat IGFBPs and the variable cross-reactivity of human antibodies to rat IGFBPs necessitated the use of ligand blotting. ^{125}I-IGF-II ligand blotting in rat kidney and liver tissue yielded six bands of IGFBPs with apparent molecular weights of 38–47 kDa (doublet), 34, 30, 24 kDa and 20 kDa. In accordance with previous observations the 38–47 kDa doublet band may correspond to the acid-stable IGF binding subunit of IGFBP-3, the 24 kDa band to IGFBP-4 and the 30 kDa band may represent both IGFPB-1 and -2, as these IGFBPs in rats have similar molecular weight [1, 8].

Induction of STZ-diabetes causes a dramatic increase in hepatic mRNA levels of IGFBP-1 and IGFBP-2, with complete or partial normalization following insulin therapy [3, 5], suggesting that the main regulators of these two IGFBPs are insulin and/or blood glucose levels [1, 3, 5]. Furthermore, circulating IGFBP-1 levels exhibit a four-fold increase as early as 2 to 3 days after induction of diabetes [4]. In addition, circulating levels of IGFBP-3 are decreased in experimental diabetes in rats after a diabetes duration of 3 weeks [8].

In the present study we demonstrated a transient increase in a 30 kDa IGFBP band in hypertrophying kidneys from diabetic rats. The increased levels were totally abolished in kidneys from insulin-treated diabetic rats, as was the renal growth. It is noteworthy that the rise in the 30 kDa IGFBPs took place in the very early stages of diabetic kidney hypertrophy and that the temporal change in the 30 kDa band is almost superimposible on the previously described transient increase in kidney IGF-I in experimental diabetes in rats [6]. A transient increase in the 30 kDa band was also observed in liver tissue from untreated diabetic animals, although this rise is not associated with a transient increase in IGF-I levels in this organ [6]. These differences in IGF-I and IGFBP levels in kidney and liver may demonstrate differential and flexible regulation of IGFs and IGFBPs in different tissues. In this context, it remains to be elucidated whether the increase in kidney and liver IGFBPs is due to increased uptake of circulating IGFBPs from serum or to increased local production.

Circulating levels of IGFBP-3 are low in experimental diabetes in rats after a diabetes duration of 3 weeks [8]. In the present study the 38–47 kDa doublet band was elevated in both kidney, liver and serum during the first 2 days after induction of diabetes, despite the fact that GH secretion is gradually decreasing within this period. This increase was followed by a subsequent decrease on day 4 in all animals in kidney, while in serum a decrease was seen only in untreated diabetic rats.

The biological roles of IGFBPs in general and in diabetes in particular are not known. The original concept of the function of IGFBPs (especially IGFBP-3) was that they act as carriers for IGFs, and that they protect IGFs from degradation, thereby ensuring a sufficient IGF supply to target tissues. Now, when it is evident that IGFs as well as IGFBPs are produced by several tissues, another dimension of IGFBP action has developed, emphasizing a role as local modulators of IGF actions. Such mechanisms may be operative for IGFBP-1 and -2, since these binding proteins, in contrast to IGFBP-3, contain a Arg-Gly-Asp tripeptide near the C-terminal, which implies that they could interact with cell surfaces and thus deliver IGF to adjacent IGF receptors, enhancing the subsequent binding and actions of IGF-I on cells [2]. Other workers have, however, found that these binding proteins inhibit IGF-I binding to some cell types [9], raising the possibility of differential local mechanisms to regulate individual tissue responsiveness to IGF-I. Finally, Busby et al. [10] reported that one form of IGFBP-1 can form multimers through disulphide linkage and thus preferentially adhere to extracellular matrices and cell surfaces. Future research in this field should be directed at elucidating the mechanisms underlying the changes in kidney and liver IGFBPs in diabetes, and searching for different growth properties of the IGFBPs.

In conclusion, the present study shows a transient increase in the 30 kDa and in the 38–47 kDa IGFBP species in hypertrophying diabetic rat kidneys. Due to molecular weight similarity between rIGFBP-1 and rIGFBP-2 the rise in this 30 kDa band may reflect a rise in either or both of these IGFBPs. It is interesting that the transient accumulation in kidney IGFBPs occurs simultaneously with the previously described transient increase in extractable kidney IGF-I in diabetic animals. This temporal relation-

ship supports the notion that IGFBPs may be involved in the renotropic action of IGF-I in diabetic kidney hypertrophy.

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