Protein glycation: its role in the changes induced by diabetes in the properties of the serum insulin-like growth factor-I binding proteins

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ABSTRACT

The purpose of this work was to study the effect of diabetes on ¹²⁵I-labelled insulin-like growth factor (IGF) binding to specific serum binding proteins (IGFBPs) and the possible role of protein glycation in such an effect. Accordingly, ligand blotting and fructosamine assays were performed in serum samples from diabetic and non-diabetic eSS rats as well as in samples of normal rat serum previously incubated with different concentrations of glucose.

IGFBPs with molecular weights of 24, 30 and 40 kDa were identified in samples from diabetic and non-diabetic rats. ¹²⁵I-Labelled IGF-I binding to each of these fractions increased significantly in the serum of diabetic rats. IGF-I binding to IGFBP-40 increased significantly as a function of the degree of glycation of serum proteins. Conversely, the increased binding of IGFBP-24 and IGFBP-30 was related only to the glucose concentration attained at 120 min during the oral glucose tolerance test.

Glycation of proteins of normal serum and the binding of labelled IGF-I increased as a function of glucose concentration in the incubation media. In these in-vitro glycated normal sera, only the binding to IGFBP-40 increased significantly; this increase was closely related to the amount of protein glycation. No clear and reproducible changes occurred with the binding of ¹²⁵I-labelled IGF-I to IGFBP-24 and IGFBP-30 fractions.

These results confirm the increase in the binding capacity of IGFBPs reported in diabetic animals. They also show that the increase in IGF-I binding to each IGFBP fraction is regulated by a different mechanism; whereas protein glycation induces changes in IGFBP-40, this mechanism does not affect the binding properties of the other two IGFBPs.

The increased binding of IGFBP might affect the availability of free IGF-I, and the consequent alterations in IGF-I-dependent metabolic processes could explain the role of this growth factor in the pathogenesis of chronic complications of diabetes.

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INTRODUCTION

It is well known that diabetic patients with poor metabolic control have increased levels of serum growth hormone (GH) (Yde, 1969; Hansen & Johansen, 1970). Under normal metabolic conditions, these high GH levels stimulated the hepatic synthesis and release of insulin-like growth factor-I (IGF-I) (Schwander, Hauri, Zapf & Froesch, 1983; Clemmons & Van Wyk, 1984) which inhibits the secretion of GH by a negative feedback mechanism (Berelowitz, Szabo, Frohman et al. 1981). In diabetic patients with poor metabolic control as well as in several animal models there is a decrease in hepatic content of IGF-I mRNA (Goldstein, Sertich, Levan & Phillip, 1988; Bornfeldt, Arnquist, Enquist et al. 1989) and in serum IGF-I levels. Consequently, the high levels of circulating GH in these patients have been attributed to a failure of the IGF-I: GH servomechanism (Flyvbjerg, 1990). On the other hand, normal (Meriméé, Gordner, Zapf & Froesch, 1984), decreased (Carlsson, Clark, Skottner & Robinson, 1989; Hall, Johansson, Povoia & Thalme, 1989) and increased (Sato, Ikeda, Miki et al. 1988) circulating IGF-I levels have been reported in diabetic patients. Such discrepancies are probably due to differences in the selection of the patient populations or to interference of IGF-binding proteins in the assay method.

IGF-I circulates complex to specific binding proteins (IGFBPs) (Ooi & Herington, 1988; Rechler & Nissley,
1990). It is assumed that IGFBPs are not merely carriers of this growth factor, but that they are also involved in control of the metabolic effects of IGF-I (Elgin, Busby & Clemmons, 1987; Rutanen, 1990). The 40 kDa complex (now referred to as rIGFBP-3) is the predominant IGFBP in the serum of normal adult rats. A minor band of 31 kDa is also present (rIGFBP-2) and is the main IGFBP component in fetal serum. Other components, such as a 29 kDa protein representing the N-terminal fragment of the 40 kDa protein, a 30 kDa (rIGFBP-1) and a 24 kDa protein, are also present in rat serum (Yvonne, Wang, Orlowski et al. 1989).

IGFBP-3 is a GH-dependent protein and a decrease in its levels has been reported in insulin-dependent diabetes mellitus (Zapf, Hauri, Waldvogel et al. 1989). The GH-independent low molecular weight IGFBP-1 undergoes a circadian rhythm and is inversely related to plasma insulin levels in both normal and diabetic patients (Baxter & Cowell, 1987; Holly, Biddlecombe, Dunger et al. 1988; Unterman, Patel, Kumar Mahathre et al. 1990). Although it has been suggested that glucose itself may regulate IGFBP-1 secretion (Cotterill, Cowell & Silink, 1989), its concentration in diabetic subjects does not correlate with the percentage of glycosylated haemoglobin A1c (HbA1c), an index of the previous degree of hyperglycaemia (Suiikkari, Koivisto, Rutanen et al. 1988; Zapf et al. 1989). To investigate the possible relationship between IGFBP and diabetes, we have studied IGFBPs in the serum of diabetic and non-diabetic eSS rats simultaneously. This strain of rats (eSS) bred in Argentina develops a spontaneous non-insulin-dependent diabetic syndrome (Tarrés, Martínez, Liborio et al. 1986; Martínez, Tarrés, Montenegro et al. 1988). We have also studied the properties of IGFBP in normal rat serum samples after its in-vitro glycation.

MATERIALS AND METHODS

Experimental animals and serum preparation

Male diabetic and non-diabetic eSS rats were used. These animals were bred in the School of Medicine of Rosario University, Rosario, Argentina. All animals were kept under a 12 h light : darkness cycle with free access to a balanced commercial diet and water.

Diagnosis of diabetes was assessed by measuring fasting serum glucose levels or by an oral glucose tolerance test (OGTT). This test was performed in each animal, except those with abnormal levels of fasting serum glucose. Fasting serum glucose levels above 7.7 mmol/l or two values ≥ 11 mmol/l during the OGTT were employed as diagnostic criteria for diabetes.

Blood samples were obtained from the retro-orbital plexus and allowed to clot. They were centrifuged (1000 g for 15 min) at 4 °C and stored at −20 °C until required. The animals were killed by decapitation under light ether anaesthesia.

Non-enzymatic glycation of serum proteins

Aliquots of normal rat serum were glycated by incubating the sample for 48 h at 37 °C in the presence of different glucose concentrations (8:3-55:5 mmol/l) and a mixture of 0.1 mmol phenylmethylsulphonyl fluoride (PMSF)/l and 1 mmol EGTA/l to inhibit protease activity. The degree of glycation was measured by the fructosamine method (Test-combination Fructosamine, Boehringer, Mannheim, Germany).

Ligand blotting

Serum samples (5 µl) were mixed with a sample buffer (62.5 mmol Tris–HCl/l, pH 6.8, 20 g sodium dodecyl sulphate (SDS)/l, 10% (v/v) glycerol and 0.01 g bromophenol blue/l) four times concentrated (Laemmli, 1970) under non-reducing conditions and boiled for 3 min before being loaded onto a 12.5% SDS–polyacrylamide gel (16 × 13 × 0.15 cm). Under these conditions, endogenous IGFs are dissociated by SDS, but the molecular weight reflects that of the binding component alone (Rechler & Nissley, 1990). Samples were electrophoresed at 200 V until the dye front reached the bottom of the gel. Prestained molecular weight marker proteins were run in a parallel lane. Proteins were transferred to nitrocellulose membranes at room temperature in Tris base (15 mmol/l)–glycine (120 mmol/l) buffer (pH 8.3) containing 5% (v/v) methanol (Hossenlopp, Seurin, Segovia-Quinson et al. 1986). Electroblotting was performed under constant current at 100 mA for 16–18 h. After the gels had been transferred, they were stained to detect any untransferred proteins. The nitrocellulose paper was blocked at 4 °C as described by Hossenlopp et al. (1986) and incubated with 125I-labelled IGF-I (2 × 105 c.p.m./ml) in 1% IGFBP-free bovine serum albumin (Boehringer)-0.1% Tween 20 in TS buffer (150 mmol NaCl/l, 10 mmol Tris–HCl/l, pH 7.4, 0.5 mmg sodium azide/ml). The specificity of 125I-labelled IGF-I binding to each IGFBP was assessed by complete displacement of the tracer by the addition of 2.5 µmol unlabelled IGF-I/l to the incubation medium (data not shown). Labelled IGF-I was kindly provided by Dr R. Gutman, Hospital Italiano, Buenos Aires, Argentina. Following this incubation, the membranes were washed, stained and autoradiographed using X-Omat Kodak film and a Dupont intensifying screen, for approximately 3 days. To measure the amount of 125I-labelled IGF bound to each IGFBP fraction, the nitrocellulose paper was cut into strips and the strips were counted in a well gamma counter. The interassay coefficient of variation for this procedure, obtained by running the same sample in six different
TABLE 1. Biological and biochemical characteristics of the eStilman Salgado (eSS) rat. Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Age (months)</th>
<th>Fasting serum glucose (mmol/l)</th>
<th>Serum insulin (pmol/l)</th>
<th>Fructosamine levels (μmol/l)</th>
</tr>
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<tr>
<td>Control</td>
<td>248 ± 32</td>
<td>6.3 ± 0.6</td>
<td>6.3 ± 0.33</td>
<td>103 ± 48</td>
<td>271 ± 14</td>
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<td>(n=9)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Diabetic</td>
<td>293 ± 16</td>
<td>9.2 ± 1.0*</td>
<td>9.3 ± 0.44**</td>
<td>248 ± 76</td>
<td>291 ± 15</td>
</tr>
<tr>
<td>(n=15)</td>
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*P<0.05, **P<0.001 compared with control (Wilcoxon rank sum test). Biochemical parameters were determined in duplicate.

gels, was 5% for IGFBP-40. Replication of results was also tested by electrophoresis of eight samples in three separate gels.

Assays

Serum glucose levels were determined by the glucose oxidase method (Glucosa GOD-PAP, Boehringer, Buenos Aires, Argentina).

Serum immunoreactive insulin was measured by radioimmunoassay using rat insulin as standard (kindly provided by Dr L. Heding, Copenhagen, Denmark), according to the method of Herbert, Lau, Gottlieb & Bleicher (1956).

The OGTT was carried out by administration of 2 g glucose/kg body weight in 10% (w/v) solution through a gastric tube. Samples of retro-orbital plexus were obtained at 0, 30, 60 and 120 min and glucose levels were determined as described above.

Statistical analysis

Statistical analysis of the data was performed by the Wilcoxon rank sum test and linear correlation analysis.

RESULTS

Table 1 shows the values of biological and biochemical parameters measured in the eSS rats included in the study. In the eSS rats, the diabetic syndrome develops with age. Hence, control animals were younger than diabetic ones. The body weight values recorded indicate that obesity was not an important characteristic of these animals.

Serum glucose levels were significantly (P<0.001) higher in the diabetic than in the non-diabetic control rats. Although not significant, serum insulin levels were also higher in the group of diabetic rats. Similarly, the glycated protein levels were higher, though not significantly, in diabetic than in non-diabetic control rats. No significant changes were observed in serum protein concentration between diabetic and non-diabetic rats (data not shown).

Figure 1 shows that the same number and molecular size of serum IGFBPs were observed in the serum of diabetic and non-diabetic eSS rats. Three bands with molecular weights of 40 (IGFBP-3), 30 and 24 kDa were identified, the first being the main component of serum IGFBP and the last the minor one.

![Figure 1](https://via.placeholder.com/150)

**Figure 1.** Ligand blotting of serum IGF-I binding proteins of non-diabetic (lane 1) and diabetic (lane 2) rats.

125I-Labelled IGF-I bound to each IGFBP fraction as well as overall binding increasing significantly (P<0.001) in the diabetic rats (Fig. 2).
IGF binding to IGFBP-3 showed a significant positive correlation with the concentration of serum glycated proteins \((P < 0.05)\) for all rats, diabetic and non-diabetic. On the other hand, the low molecular size fractions (30 and 24 kDa) showed a positive correlation with the 120-min glucose levels of the OGTT \((P < 0.05)\). These results suggest that the non-enzymatic glycation of serum proteins which occurs in the diabetic animals could affect the binding of \(^{125}\text{I}-\text{IGF-I}\) to the IGFBP. To test this hypothesis, we performed a ligand blotting analysis of IGFBP in a pool of normal rat serum previously incubated with different glucose concentrations. The degree of protein glycation in this serum increased as a function of the glucose concentration in the incubation medium. The fructosamine values measured in the incubated serum were within the range of the values found in the serum samples of the diabetic animals. Resembling the phenomenon observed in the serum of the diabetic rats, in the in-vitro glycated serum the binding of labelled IGF to IGFBP-3 increased significantly (Fig. 3); the magnitude of this increment was positively related to the degree of glycation of the serum proteins (Fig. 4). Although binding of the tracer to IGFBP-24 and IGFBP-30 also changed, the changes were not related to the concentration of glycated protein in the serum samples.

**DISCUSSION**

Serum IGFBPs were studied in diabetic and non-diabetic eSS rats. The diabetic syndrome of these rats has the characteristics of a non-insulin-dependent type with a slow progressive and mild course, allowing the animal a survival period of more than 18 months without insulin treatment. During such a period the animals develop a chronological sequence of islet alterations as well as chronic lesions in the kidney, peripheral nerves and striated muscle (Gagliardino, Gómez Dumm, Semino et al. 1989; Gómez Dumm, Semino & Gagliardino, 1990). The diabetic animals included in our study showed mild fasting hyperglycaemic values, a non-significant increase in serum immunoreactive insulin and a slight increase in serum fructosamine levels.

Ligand blotting analysis of the serum samples showed the same IGFBP pattern in both groups of rats with 40, 30 and 24 kDa bands, the first one being the major and the last one the minor component.

Binding of \(^{125}\text{I}-\text{IGF-I}\) to each one of these fractions was significantly increased in serum samples from diabetic animals. However, the increased binding of labelled IGF-I to each fraction was not equally related to the same parameter of carbohydrate metabolism. In the whole group of rats, diabetic and non-diabetic, binding of IGFBP-3 (40 kDa) increased significantly as a function of serum fructosamine levels, while IGFBP-30 and -24 binding was related only to the glucose values attained at 120 min after the oral glucose load. These results suggest that, while the increased binding of IGFBP-3 probably depends on the glycation of the IGFBP-3 molecule, the corresponding binding to IGFBP-24 and -30 reflects another abnormality of the metabolism of glucose present in diabetic rats.

Fructosamine levels, in the wide range from normal to diabetic values, significantly affected the magnitude of IGF-I binding to IGFBP-3. Hence, subtle glycation near IGF-I binding sites of IGFBP-3 might be enough to modify its binding properties. This could explain why the non-significant increase in fructosamine concentration was still capable of increasing IGFBP-3 binding significantly. Normal rat serum incubated with different concentrations of glucose showed a dose-dependent glycation of its proteins. Under this condition, only IGFBP-3 binding was significantly increased and positively related to the fructosamine level. No consistent changes were observed in IGF-I binding to IGFBP-24 and IGFBP-30. These results support our above hypothesis regarding the modulatory effect of protein glycation on the IGF-I binding of IGFBP-3, while IGFBP-24 and IGFBP-30 binding properties may be influenced by other diabetic hormonal or metabolic alterations. Similar to our findings, an increase in the overall binding of IGF-I to serum IGFBP has been reported in rats made diabetic by streptozotocin (Unterman et al. 1990). This increase, however, was ascribed by the authors to the low molecular weight component of the IGFBP. It has been reported that the secretion of this low molecular weight IGFBP does not correlate with the percentage of glycated haemoglobin (Suikkari et al. 1988; Zapf et al. 1989). On the other hand, the serum concentration of this IGFBP fraction showed a negative relationship with the circulating levels of insulin (Baxter & Cowell,
FIGURE 3. Ligand blotting analysis of IGF-I binding proteins of rat serum incubated at 37 °C for 48 h with different glucose concentrations. Levels of fructosamine (µmol/l) were: lane 1, 190; lane 2, 334; lane 3, 622.

FIGURE 4. Relationship between 125I-labelled IGF-I bound to IGF-I-binding protein-3 (IGFBP-3) and fructosamine levels in in-vitro glycated normal rat serum. r = 0.98; P < 0.001.

1987). Hence, the insulin levels measured in the serum of our diabetic eSS rats, which are opposite to the low values found after streptozotocin-induced diabetes, as well as the results obtained in vitro, could explain the apparent discrepancy between the report of Unterman et al. (1990) and the current data.

Our results confirm the increase in the 125I-labelled IGF-I to IGFBP in diabetic animals. They also show, in agreement with the results of Holly, Smith, Dunger et al. (1989), that the increase in the IGF-I binding to each IGFBP fraction is produced by different mechanisms. While protein glycation would induce the changes in IGFBP-3, this mechanism would not affect the binding properties of IGFBP-24 and IGFBP-30. Our results cannot explain the intrinsic mechanism by which glycation produces this effect.

The increased binding by IGFBP in diabetic animals could affect the amount of serum free IGF-I and consequently impair IGF-dependent processes. This growth factor modulates multiple metabolic pathways in several tissues (Van Wyk, 1984). Therefore, an alteration in the free : bound ratio of IGF could explain, at least partly, the role claimed for IGF-I in the pathogenesis of chronic complications of diabetes (Baumann, 1990).

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