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RECEPTORS AND BINDING PROTEINS FOR INSULIN AND INSULIN-LIKE GROWTH FACTORS IN THE PLACENTA OF HEALTHY MOTHERS AND MOTHERS WITH INSULIN-DEPENDENT DIABETES MELLITUS

RECEPTORI I VEZUJUĆI PROTEINI ZA INSULIN I INSULINU SLIČNE FAKTORE RASTA U PLACENTI ZDRAVIH MAJKI I MAJKI SA INSULIN-ZAVISNIM DIJABETES MELITUSOM

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Summary: The IGF system of human placenta consists of insulin-like growth factors (IGF)-I and -II, their receptors (IGF-1R and IGF-2R), and binding proteins (IGFBP-1 to -6). Due to many structural and metabolic similarities with insulin, the IGF system cannot be examined separately from insulin and its receptor (IR). In this study gel filtration was used to detect solubilized membrane proteins of the placenta obtained from healthy mothers and mothers with IDDM. In order to detect placental membrane proteins that bind IGF molecules (and insulin), the solubilized membranes were incubated with each of the three ^{125}I-labelled ligands: ^{125}I-IGF-I, ^{125}I-IGF-II and ^{125}I-insulin prior to gel filtration chromatography. The biochemical evidence of the presence of receptors for insulin and IGFs, as well as that of IGFBP-1 were obtained by immunoblotting. Herein we demonstrated that, considering IGF and insulin receptor content, the placental tissue obtained from mothers with IDDM was not different from that obtained from healthy mothers. However, the concentration of IGFBP-1 differed between the examined placentas. IDDM in mothers caused an increase in the amount of IGFBP-1 in their placentas and, consequently, the amount of the labelled ligand bound to it. The redistribution of IGFs between the receptors and IGFBP-1 may be involved in regulatory mechanisms in the placenta of mothers with IDDM.

Keywords: diabetes, IGFBPs, IGF-1R, IGF-2R, IR, placenta

Kratak sadržaj: IGF sistem humane placente sačinjavaju insulinu slični faktori rasta (IGF)-I i -II, receptori za koje se oni vezuju (IGF-1R i IGF-2R), kao i njihovi vezujući proteini (IGFBP-1 do -6). Usled strukturnih i metaboličkih sličnosti sa insulinom, IGF sistem se ne može izučavati odvojeno od insulina i njegovog receptora (IR). U ovom radu gel filtracija je korišćena kako bi se detektovali membranski proteini iz solubilizata membrana dobijenih iz placenti zdravih majki i majki sa IDDM. Pojedinačni uzorci solubilizata preinkubirani su sa tri ^{125}I-liganda: ^{125}I-IGF-I, ^{125}I-IGF-II ili ^{125}5I-insulinom, a zatim hromatografisani. Biohemijski dokazi prisustva receptora za insulin i insulinu sličnih faktora rasta, kao i IGFBP-1 dobijeni su imunoblotingom. Rezultati su pokazali da se tkiva placenti dijabetičnih majki ne razlikuju od zdravih u pogledu IGF i insulinskog receptora, ali razlike postoje na nivou IGFBP-1. IDDM izaziva povećanje količine IGFBP-1 i, shodno tome, povećanje količine liganda koji se vezuje za njega. Ova preraspodela IGF molekula između receptora i IGFBP-1 može učestvovati u regulatornim mehanizmima u placenti majki sa IDDM.

Ključne reči: dijabetes, IGFBP, IGF-1R, IGF-2R, IR, placenta

Introduction

The biological actions of insulin-like growth factors (IGFs) include profound effects on growth, differentiation, survival and metabolism in almost all cell types (1, 2). Although it is well known that IGFs are mediators of fetal growth (3), the processes that control human placental growth are poorly understood (4). IGF-I and IGF-II are two small, highly homologous peptides of approximately 7.5 kDa, structurally

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similar to proinsulin (5). The biological actions of both IGF molecules are mediated mainly through interactions with the type 1 IGF receptor (IGF-1R) (3, 6). In addition, IGF-II binds to the type 2 IGF receptor (IGF-2R), which is also a physiological receptor for mannose-6-phosphate (M6P) and M6P-bearing lysosomal enzymes (7), and to the insulin receptor isoform A (8).

Distinct from other growth factors, IGFs are known to associate with specific binding proteins called IGF binding proteins (IGFBPs) in plasma and tissues, of which six have been characterised to date (9–11). IGFs and IGFBPs form macromolecular IGF/IGFBP complexes in the circulation, which prolong the half-lives of IGFs (10). Locally expressed IGFBPs often serve as a means of attracting IGFs in specific cells or manipulating local IGF biological activities of IGFs (12). Although IGFBPs share relatively high amino acid sequence homology among themselves, each of them possesses unique structural and biochemical properties and is subject to tissue-specific expression and hormonal regulation.

IGFs bind to the soluble forms of IGFBPs with either equal or higher affinity than to the IGF receptors, allowing these binding proteins to modulate the biological activity of IGFs and thus regulate its interaction with IGFRs in a tissue-specific fashion (12). Whereas some IGFBPs have been shown to inhibit IGF actions by preventing them from gaining access to the IGF receptors, others potentiate IGF actions by facilitating the ligand receptor interaction (13). Beside these inhibitory and stimulatory effects of IGFBPs on IGF actions, the existence of a third set of IGFBP actions, those classed as IGF-independent actions, has become evident. These actions are more accurately described as IGF-1R independent and are likely to involve structural domains of IGFBPs that are distinct from the IGF-binding determinants (11). As an example, $\alpha_5\beta_1$-integrin binding by IGFBP-1, which has the Arg-Gly-Asp integrin binding motif in its sequence, was found to be responsible for human trophoblast cell migration (14).

The human placenta can synthesize IGF-I and IGF-II from an early stage of gestation (15). IGF-1R, IGF-2R and IR are abundantly found in the human placenta (16, 17). Human placental cells do not express IGFBP-1, yet it is the most abundant binding protein in the decidualized endometrium (18). The association of IGFBP-1 with the placental cell membranes was also reported (19, 20). Post-translational modifications have been shown to alter the affinity of IGFBP-1 for IGF-I, including phosphorylation (21), proteolysis (22), and polymerization (23). In the circulation of non-pregnant women IGFBP-1 almost exclusively exists in its phosphorylated form, which has high affinity for IGFs. During pregnancy however, IGFBP-1 becomes dephosphorylated by placental alkaline phosphatase to non-phosphorylated and lesser phosphorylated isoforms, which have reduced affinity for IGF-I (21).

Insulin has an important role in regulating IGF bioactivity through inverse stimulation of hepatic IGFBP-1 production (24). On the other hand, IGFs and IGFBPs participate in glucose metabolism and homeostasis (25). The IGF system is, therefore, so closely entangled with insulin and its receptor that they are often examined together.

The aim of this work was to compare the presence of some of the components of the IGF/insulin system, namely IGF-1R, IGF-2R, IR and IGFBP-1, in the solubilized placental membranes obtained from healthy mothers and mothers having insulin-dependent diabetes mellitus (IDDM, diabetes type 1).

Materials and Methods

Ligands

$Na^{125}I$ was supplied by Isotope (Budapest, Hungary). Porcine insulin was from NOVO (Copenhagen, Denmark). Human IGF-I and IGF-II were from ICN Biochemicals (Costa Mesa, CA, USA). ^{125}I-IGF-I, ^{125}I-IGF-II and ^{125}I-insulin were prepared using the chloramine T method (26). The specific activities were approximately 100 μCi/μg.

Placentas and membrane solubilisates

Human placentas (n=6) were obtained after normal, full-term delivery from three healthy and three diabetic mothers, with the approval of the local ethical committee. All diabetic mothers were diagnosed as IDDM class B, having diabetes for 4–8 years and they were on insulin therapy. The relative amount of the glycosylated haemoglobin in their blood was below 6.2 % and these mothers were assumed to be »well-regulated«.

The tissue was collected in ice cold 0.1 mol/L phosphate buffered saline (pH 7.4), brought to laboratory within 60 min and washed free of blood. The amniotic and chorionic membranes and the large blood vessels were dissected away. Placental tissue was minced and homogenised in a 0.25 M sucrose solution supplemented with protease inhibitors. After a 10 min centrifugation at 600 g the pellet was discarded. The supernatant was centrifuged at 18000 g for 30 min. The resulting pellet was washed once in 0.5 mol/L HEPES buffered saline (pH 7.4) and resuspended in the same buffer. Solubilization was performed by adding Triton X-100 into the membrane suspension at the final concentration of 1% with stirring for 1 h at 4 °C. After centrifuging the suspension at 100 000 g for 90 min at 4 °C, the supernatant was divided into aliquots and stored frozen at – 50 °C until used. The protein concentration in the solubilized membranes was determined by the method of Bradford (27).

Gel filtration

Solubilized membranes (2 mg of membrane protein) were incubated with ^{125}I-IGF-I, ^{125}I-IGF-II or ^{125}I-insulin (1 pmol, 10^6 cpm) at 4 °C overnight. The samples were then chromatographed using 0.5 mol/L sodium phosphate buffer pH 7.5 containing 0.1 mol/L NaCl and 1 % Triton X-100 (v/v) on a Sephadex G-100 column (1.8 x 60 cm). The flow rate was 20 mL/h. Fractions of 1.5 mL were collected and their radioactivity measured. Experiments were performed in triplicate. Ligand binding to different membrane proteins was expressed as the percentage of the total radioactivity eluted and was used as a measure of receptor and IGFBP abundance in the solubilised membranes for each ^{125}I-ligand (28).

Electrophoresis

Solubilized membrane proteins were analysed by the native electrophoresis (nPAGE) using 6% gel, under non-reducing conditions (29). Resolved proteins were transferred to Immobilon membranes and immunoblotted with the primary antibodies: anti-IGF-1R, anti-IGF-2R (Biosource, Camarillo, CA, USA), anti-IR (GroPep, Adelaide, Australia) and anti-IGFBP-1 (DSL, Webster, TX, USA). After incubation with the secondary antibodies, membranes were treated with the ECL detection kit (Amersham Biosciences, Uppsala, Sweden) and autoradiographed using X-ray film (Kodak, Cedex, France).

Statistical analysis

The differences in the results, expressed as means ($\bar{x}$) ± standard deviation (SD), between healthy mothers and mothers with IDDM were evaluated using the Student's t test.

Results

Gel filtration was used for detection of all molecular species from the solubilized membranes that interacted with ^{125}I-IGF-I, ^{125}I-IGF-II or ^{125}I-insulin (28). Three representative elution profiles, one for each ^{125}I-labelled ligand, are shown in *Figure 1*.

The placental cell membrane proteins that bound ^{125}I-IGF-I were resolved into two radioactive peaks upon gel filtration: the first peak (peak I) appeared at the void volume (V_0) of the column and represented the IGF-1R, the presence of which was further confirmed by immunoblotting (*Figure 2*). The second peak eluted at the V_e of ovalbumin (45 kDa) and corresponded to IGFBP-1 (28). The identity of IGFBP-1 was also documented by immunoblotting (*Figure 2*). The third peak represented the free radioligand. ^{125}I-IGF-II- binding proteins were also eluted in two peaks, with a profile similar to that of the ^{125}I-IGF-I. The peak positions were the same, but the height of peak I was smaller, reflecting a lower content of the ^{125}I-IGF-II-binding receptors. The presence of IGF-2R was, again, confirmed by immunoblotting (*Figure 2*). To the contrary, gel filtration of the solubilised membranes preincubated with ^{125}I-insulin

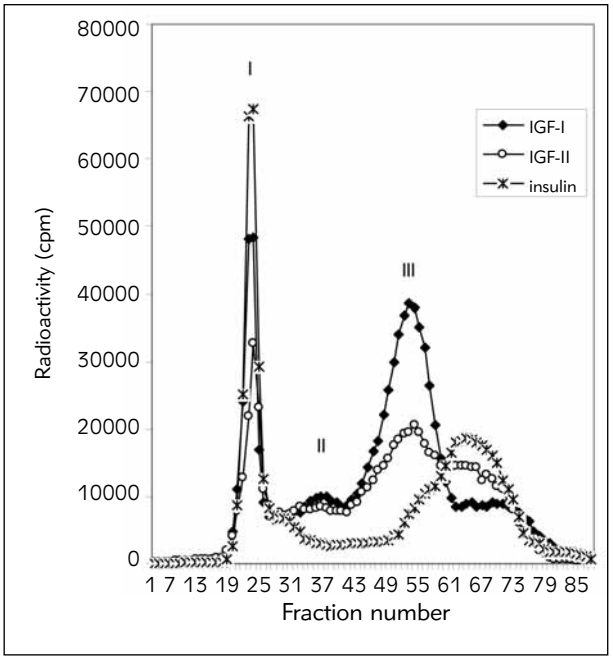

Figure 1 Gel filtration of the solubilised placental membranes preincubated with ^{125}I-IGF-I, ^{125}I-IGF-II or ^{125}I-insulin (a representative elution profile).

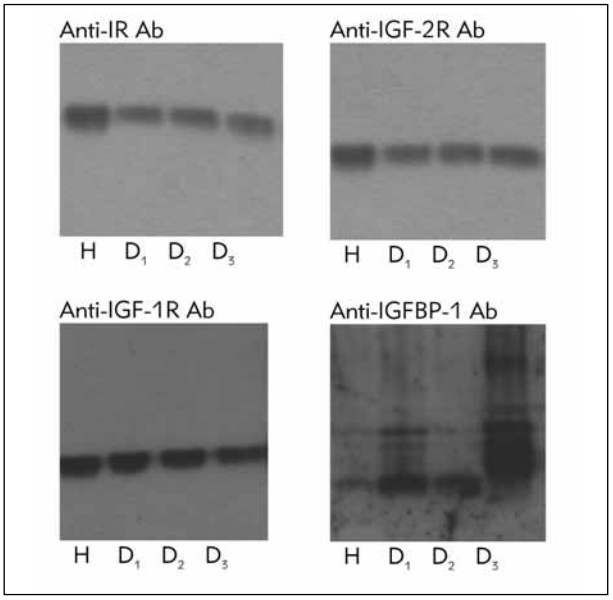

Figure 2 Native electrophoresis of the solubilised placental membranes and immunoblotting with anti-IR, anti-IGF-1R, anti-IGF-2R and anti-IGFBP-1 antibodies (H: healthy mother, D_1, D_2 and D_3: diabetic mothers).

Table I The relative content of IGF-1R, IGF-2R, IR and IGFBP-1 in the solubilised placental membranes of healthy mothers and mothers with IDDM.

The relative content	Placenta from healthy mothers (n = 3)	Placenta from IDDM mothers (n = 3)
The amount of proteins in peak I compared to the total amount of proteins in the solubilisate (%)	25.5 ± 4.95	33.0 ±5.28
The amount of proteins in peak II compared to the total amount of proteins in the solubilisate (%)	1.0 ± 0.27	3.05 ± 0.92*
Relative ratio of the three receptor types IR : IGF-1R : IGF-2R	4 : 3 : 2	4 : 3 : 2
Relative ratio of the radioactivities in protein peaks I : II (^{125}I-IGF-I as a ligand)	5.95 ± 0.75	1.56 ± 0.36*
Relative ratio of the radioactivities in protein peaks I : II (125I-IGF-II as a ligand)	6.15 ± 0.98	3.32 ± 0.95*

Results are presented as means ($\bar{x}$) and the standard deviation (SD). Statistically significant differences between two groups are indicated as $p<0.05$ (*).

produced a profile lacking a peak that included IGFBP-1. The presence of IR was proven using anti-IR antibody in blotting (*Figure 2*).

By measuring the surface area under the peaks, the relative presence of IGFRs and IR, as well as IGFBP-1, was calculated for the solubilized placental membranes obtained from healthy and diabetic mothers. These data were used to estimate the relative ratio of the three types of receptors, as well as the ratio between the amount of ligand bound to the specific receptor and to IGFBP-1. The results are shown in *Table I*.

Having determined the relative amount of IGF and insulin binding structures in the placental membranes, we sought the possible existence of molecular isoforms of the IGF-1R, IGF-2R, IR and IGFBP-1 using nPAGE followed by immunoblotting. As can be seen from the *Figure 2*, all receptors migrated as a single protein band in the electrophoretic gel. In contrast, IGFBP-1 split into several protein bands, each reflecting a different isoform (29). No difference was observed between the analysed samples with respect to IGF and insulin receptor forms, but the samples differed with respect to IGFBP-1. Whereas in some membrane preparations only one distinct IGFBP-1 protein band was detected, up to five different isoforms were found in the others. The band intensity varied from major to weak intensity, reflecting the different content of individual IGFBP-1 isoforms in the analysed samples.

Discussion

The membranes of human placental cells possess specific binding sites for IGF-I, IGF-II and insulin (16). These cells also produce different IGFBPs (18). Having affinities for IGF molecules that are equal to or even greater than those of the IGF receptors (12), IGFBPs are potent modulators of IGF actions. Among the binding proteins, IGFBP-1 is the predominant IGF carrier during fetal life (15), so in this study it was examined in parallel with the receptors.

IGFBP-1 is transcriptionally repressed by insulin and its circulatory concentration displays a diurnal variation in response to changes in the insulin concentration (30). Furthermore, it was shown that insulinopenia of IDDM affects the synthesis of IGFBP-1 (31). Since »ready-to-use« IGF-I circulates in binary complexes with IGFBP-1 and since only the unbound form of IGF is suggested to be biologically active, the significance of insulin, through its regulative role in IGFBP-1 production, in determining the bioavailability of IGF- I cannot be neglected. Brismar and colleagues demonstrated the increase in serum IGF-I concentration following insulin infusion in patients with IDDM that was provoked by a drop in the IGFBP-1 level (31). The physiological secretion of insulin and its exogenous application by infusion differ not only in the total amount of the hormone but in the rapid oscillations in insulin secretion (a characteristic of functional pancreas) and in the rate of its clearance, so that even »well-controlled« IDDM may cause altered IGFBP-1 production.

Post-translational modifications of proteins may significantly alter their physical characteristics, such as charge and conformation, and biochemical characteristics which include interactions with other molecules. Ligand-binding affinity, proteolytic stability and nearly entire cell-signalling pathways depend on the type and degree of post-translational protein modifications.

IGF and insulin receptors of the human placenta were found to be differentially glycosylated (28). IGFBP-1 has been found to be phosphorylated and four to five phosphorylated isoforms and one non-phosphorylated isoform have been identified in different biological fluids. Non-phosphorylated IGFBP-1 has 4- to 6-fold lower affinity for IGF-I compared to

that of phosphorylated IGFBP-1 (32). Phosphorylated forms inhibit, whereas the non-phosphorylated form enhances IGF-I action in cultured trophoblast cells (33). The phosphorylation state of IGFBP-1 is altered during pregnancy, which is characterised by the increase in non-phosphorylated IGFBP-1 and the appearance of less phosphorylated IGFBP-1 compared to those in plasma from non-pregnant women (21). This suggests that changes in the IGFBP-1 isoform pattern in the mother may influence fetal growth, as well as changes in total IGFBP-1, as non-phosphorylated and phosphorylated IGFBP-1 may differently modify IGF-I action in the placenta.

The results of this study demonstrated that placental tissues obtained from mothers with IDDM did not differ from the tissues obtained from healthy mothers in respect to IGF and insulin receptors, but alteration was found at the IGFBP-1 level. Two groups of solubilized placental membranes had similar amounts of proteins in the receptor fraction (approximately 30% of the total amount of proteins in the solubilisate) and the relative ratio of the three types of receptors was kept constant regardless of IDDM. Immunoblot analysis also showed no difference between the two study groups, concerning both the amount and the mobility of IGF-1R, IGF-2R and IR molecules.

Placental membrane solubilisates from IDDM mothers contained approximately three times more proteins in the IGFBP-1 region than those of healthy mothers. Increased amount of IGFBP-1 bound more ^{125}I-labelled ligand, which resulted in a shift of the ratio of the ligand bound to the receptor fraction and IGFBP-1 fraction. Six times more ligand was bound to the receptors than to IGFBP-1 when the solubilisates from healthy mothers were examined. Two to three times more ligand was bound to the receptors than to IGFBP-1 from the solubilisates of IDDM mothers. The immunoblot analysis revealed that IGFBP-1 was present in several molecular isoforms and the isoform pattern differed between the samples obtained from healthy and IDDM mothers, but without regularity that would allow making firm conclusions.

Although the number of cases included in this study was small, it can be concluded that IDDM may not necessarily cause the amount of IGF and insulin receptors or their affinity for the ligands to change. On the other hand, IDDM did cause an increase in the amount of IGFBP-1 and, consequently, the amount of ligand that binds to it. Redistribution of IGF molecules between their receptors and IGFBP-1 may be involved in some of the regulatory mechanisms in the placental tissue of mothers with IDDM.

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