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N-GLYCOSYLATION PATTERN OF HUMAN PLACENTAL INSULIN-LIKE GROWTH FACTOR AND INSULIN RECEPTORS IN WELL-CONTROLLED PREGESTATIONAL DIABETES MELLITUS

N-GLIKOZILOVANJE RECEPTORA ZA INSULIN I INSULINU SLIČNE FAKTORE RASTA IZ HUMANE PLACENTE KOD MAJKI SA DOBRO KONTROLISANIM PREGESTACIJSKIM DIJABETESOM

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Summary: Diabetes mellitus is a complex disease that leads to alterations in the glycosylation of proteins. Insulin-like growth factor and insulin receptors are involved in the regulation of fetal and placental growth and development. In this work the N-glycans of these receptors, originating from placentas obtained from pregnancies complicated by pregestational insulin dependent diabetes mellitus, were studied. Diabetic mothers were under regular insulin therapy. Solubilised membrane samples from healthy and diabetic placentas were analysed using lectin-affinity chromatography. N-glycans bound to insulin-like growth factor and insulin receptors were studied in terms of their interaction with eleven agarose--immobilised lectins: wheat germ agglutinin, succinylated wheat germ agglutinin, *Ricinus communis* agglutinin I, *Sambucus nigra* agglutinin, *Erythrina cristagalli* lectin, *Ulex europaeus* agglutinin, *Lens culinaris* agglutinin, *Canavalia ensiformis* lectin, *Phaseolus vulgaris* erythro- and leukoagglutinin and *Maackia amurensis* agglutinin. A very similar type of N-glycans and content of the terminal saccharide residues were found in both groups of placentas. The results of this work suggest that the tight glycemic control may prevent alterations in the glycosylation of insulin-like growth factor and insulin receptors, thus maintaining physiological homeostasis during pregnancy and fetal growth.

Keywords: diabetes mellitus, insulin-like growth factor receptors, insulin receptor, N-glycosylation, placenta

Kratak sadržaj: Dijabetes melitus je kompleksno oboljenje koje uzrokuje promene u načinu glikozilovanja proteina. Receptori za insulin i insulinu slične faktore rasta učestvuju u regulaciji rasta i razvitka fetusa i placente. U ovom radu ispitivani su N-glikani vezani kovalentnom vezom za pomenute receptore. Receptori za insulin i insulinu slične faktore rasta dobijeni su iz placenti majki sa pregestacijskim insulin-zavisnim dijabetesom. Dijabetične majke bile su na redovnoj terapiji insulinom. Uzorci ćelijskih membrana, izolovani i solubilizovani iz placenti zdravih majki i majki sa dijabetesom, analizirani su primenom lektinske afinitetne hromatografije. Ispitivana je interakcija N-glikana vezanih za receptore sa jedanaest različitih lektina, imobilisanih na agaroznom gelu: lektin iz pšeničnih klica, sukcinilovani lektin iz pšeničnih klica, *Ricinus communis* lektin I, lektin iz kore zove, lektin iz koralnog drveta, lektin iz *Ulex europaeus*-a, lektin iz sočiva, konkanavalin A, dva lektina iz pasulja i lektin iz *Maackia amurensis*-a. Rezultati ovog rada pokazali su da receptori za insulin i insulinu slične faktore rasta poreklom iz humane placente zdravih majki i onih sa pregestacijskim dijabetesom imaju veoma sličan tip N-glikana kao i sastav terminalnih ostataka saharida, što ukazuje na to da se dobrom kontrolom glikemije kod trudnica sa dijabetesom mogu sprečiti promene u načinu glikozilovanja ovih receptora, čime bi se pomoglo održavanju fiziološke homeostaze za vreme rasta fetusa.

Ključne reči: dijabetes, receptori za insulinu slične faktore rasta, insulinski receptor, N-glikozilovanje, placenta

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List of abbreviations: BMI, body mass index; Con A, Concanavalin A; DP, diabetic placenta; ECL, Erythrina cristagalli lectin; Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; HbA1c, glycated hemoglobin; HP, healthy placenta;

IDDM, insulin dependent diabetes mellitus; IGF, insulin-like growth factor; IGF1R, type 1 insulin-like growth factor 1 receptor; IGF2R, type 2 insulin-like growth factor receptor; IgG, immunoglobulin G; IR, insulin receptor; Lac, lactose; LCA, Lens culinaris agglutinin; MAA, Maackia amurensis agglutinin; Man, mannose; α-MM, methyl-α-mannopyranoside; PHA, phytohemagglutinin; RCA, Ricinus communis agglutinin; Sia, sialic acid; SD, standard deviation; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SNA, Sambucus nigra agglutinin; s-WGA, succinylated wheat germ agglutinin; TX-100, Triton X-100; UEA, Ulex europaeus agglutinin; WGA, wheat germ agglutinin.

Introduction

The insulin-like growth factor (IGF) system is comprised of two peptides (IGF-I and IGFII), cell-surface receptors and six IGF-binding proteins (1). IGF-I and IGF-II are involved in the regulation of various cellular processes, such as cellular growth, proliferation, differentiation, surviving against apoptosis and migration (2). Both IGFs are believed to signal through the type 1 IGF receptor (IGF1R), although IGF-II exerts some effects after its binding to the insulin receptor (IR) (3). IGF1R and IR belong to a family of tyrosine kinase receptors, consisting of two and two α and two β subunits with a molecular mass of approximately 420 kDa (1). The type 2 IGF receptor (IGF2R) is a single-chain polypeptide of approximately 300 kDa, which is without known signalling potency (4).

The IGF system is involved in the regulation of fetal and placental growth and development (5). IGF and insulin receptors are abundantly expressed on distinct placental cell surfaces (6). These receptors are heavily glycosylated (7). We have previously found certain differences in glycosylation between IGF and insulin receptors isolated from placentas of healthy mothers (8). The aim of this study was to investigate the N-glycans of IGF and insulin receptors originating from placentas of mothers with pregestational insulin dependent diabetes mellitus (IDDM).

Materials and Methods

Human placentas were obtained following normal full-term deliveries from five healthy and five diabetic mothers. All the subjects provided informed consent and the study was approved by the local Ethics Committee of the Institute for the Application of Nuclear Energy, Belgrade. The mothers were 27 to 32 years old and their body mass index (BMI) was 26.2 to 30.1 kg/m^2. The diabetic mothers were diagnosed with IDDM 4 to 7 years before the conception and showed neither vascular nor renal disease. All five of them had well-controlled diabetes and were regularly treated with Actrapid/Insulatard therapy. The concentration of glycated hemoglobin (HbA1c) in their sera was checked monthly during pregnancy and was in the range from 5.0 to 6.1%.

The solubilised membrane preparation of human placental IGF and insulin receptors was obtained following the procedure described in the work of Masnikosa et al. (8, 9).

Human recombinant insulin (Novo, Copenhagen, Denmark), des(1-3)-IGF-I and IGFII (GroPep, Adelaide, Australia) were labelled with ^{125}I by the chloramine T method (10) to the specific activity of approximately 100 μci/μg. ^{125}I-ligands were used within three weeks.

The presence of specific receptors was determined by immunoblotting. Monoclonal anti-IR (GroPep Limited, Adelaide, Australia), anti-IGF1R (BioSource International, Inc., Camarillo, USA) and anti-IGF2R antibody (Calbiochem, Darmstadt, Germany) were used as primary antibodies. Anti-IR and anti-IGF1R were used at a dilution of 1:400 and anti-IGF2R was used at a dilution of 1:2,000. The polyclonal goat anti-mouse IgG coupled to horseradish peroxidase (1:10,000 dilution) was used as the secondary antibody (BioSource International, Inc., Camarillo, USA). Receptor visualisation was achieved using an enhanced chemiluminescence reagent kit (Pierce, Minneapolis, USA) followed by autoradiography (Kodak MXB film and developing reagents, Paris, France).

Lectin-affinity chromatography was performed using mini columns (2 mL of gel) filled with eleven different agarose-immobilised lectins: WGA (wheat germ agglutinin), succinylated WGA (s-WGA), RCA I (*Ricinus communis* agglutinin I), SNA (*Sambucus nigra* agglutinin), ECL (*Erythrina cristagalli* lectin), UEA I (*Ulex europaeus* agglutinin I), LCA (*Lens culinaris* agglutinin), Con A (lectin from *Canavalia ensiformis*), PHA-E (*Phaseolus vulgaris* erythroagglutinin), PHA-L (*Phaseolus vulgaris* leukoagglutinin) and MAA (*Maackia amurensis* agglutinin). All gels were purchased from Vector (Vector Laboratories Inc., Burlingame, USA), except MAA, which was bought from Gentaur (Gentaur, Kampenhout, Belgium). Lactose (Lac) was from Merck (Darmstadt, Germany). Methyl-α-mannopyranoside (MM), mannose (Man), fucose (Fuc), N-acetyl-glucosamine (GlcNAc) and HEPES were from Sigma-Aldrich (Steinheim, Germany). The saccharide specificities of used lectins are given in datasheets. Before being applied to lectin columns, solubilisate samples (0.1 mL) were incubated with ^{125}I-labelled ligands (5 x 10^5 cpm) in a total volume of 1 mL of appropriate wash buffer, overnight at 4 °C. The samples were recirculated through the columns for 1 h to ensure maximal binding. The flow-through and the unbound material (which was washed away with 30 mL of the corresponding wash buffer) were combined and their radioactivity was measured in a γ-counter (non-bound radioactivity). The elution of bound complexes was performed with 7 mL of hapten sugar solution in 0.1 mol/L acetic acid, pH 3.0 (elution solution), except for PHA-E, PHA-L and MAA. The composition of wash buffers and elution

Table I Conditions for lectin-affinity chromatography.

Lectin-affinity column	Wash buffer	Elution solution
WGA-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, pH 7.5, 0.1% TX-100	0.5 mol/L GlcNAc in 0.1 mol/L acetic acid, pH 3.0
s-WGA-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, pH 7.5, 0.1% TX-100	0.5 mol/L GlcNAc in 0.1 mol/L acetic acid, pH 3.0
RCAI-agarose	RCA 0.01 mol/L HEPES, 0.15 mol/L NaCl, pH 7.5, 0.1% TX-100	0.2 mol/L Lac in 0.1 mol/L acetic acid, pH 3.0
SNA-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L Ca^{2+}, pH 7.5, 0.1% TX-100	0.5 mol/L Lac in 0.1 mol/L acetic acid, pH 3.0
ECL-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L Ca^{2+}, pH 7.5, 0.1% TX-100	0.2 mol/L Lac in 0.1 mol/L acetic acid, pH 3.0
UEA I-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L Ca^{2+}, pH 7.5, 0.1% TX-100	0.1 mol/L Fuc in 0.1 mol/L acetic acid, pH 3.0
LCA-agarose	0.01 mol/L HEPES, 0.1 mmol/L Ca^{2+}, 0.01 mmol/L Mn^{2+}, pH 7.5, 0.1% TX-100	0.1 mol/L α-MM in 0.1 mol/L acetic acid, pH 3.0
Con A-agarose	0.02 mol/L HEPES, 0.5 mol/L NaCl, 1 mmol/L Ca^{2+}, $Mg^{2+}$and Mn^{2+}, pH 7.5, 0.1% TX-100	0.3 mol/L Man in 0.1 mol/L acetic acid, pH 3.0
PHA-E-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L Ca^{2+}, 0.01 mmol/L Mn^{2+}, pH 8.0, 0.1% TX-100	0.1 mol/L acetic acid, pH 3.0
PHA-L-agarose	0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L Ca^{2+}, 0.01 mmol/L Mn^{2+}, pH 7.5, 0.1% TX-100	0.1 mol/L acetic acid, pH 3.0
MAA-agarose	0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.2–7.4, 0.1% TX-100	0.1 mol/L glycine buffer, pH 3.5

solutions is given in *Table I*. The elution conditions were either suggested by the resin producers or determined in our preliminary experiments. The experiments were performed in triplicate. Fractions (1 mL) eluted by the elution solutions were collected and their radioactivity was measured. The sum of eluted radioactivity was designated as lectin-bound radioactivity. It was expressed as a percentage of the total radioactivity applied to each agarose-bound lectin (non-bound + lectin-bound radioactivity).

After lectin-affinity chromatography of the individual placental samples, two separate pools were made using placentas from women with IDDM and placentas obtained from healthy women. The two pools of placentas were separately applied to the eleven lectin columns (without prior incubation with ^{125}I-ligands) and subjected to lectin chromatography as described. The fractions that corresponded to those with the highest binding of ^{125}I-ligand were collected from each column, pooled and immediately neutralised using 2 mol/L Tris-HCl pH 8.9. The pools were dialysed first against distilled water for 3 h at room temperature and then against 150 mmol/L NaCl overnight at 4 °C. After being concentrated to approximately 0.5 mL using a Microcon centrifugal filter device (Millipore, Billerica, MA, USA) with a 10 kDa cut-off membrane, the samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 6% gel.

Numerical results were expressed as the mean ± SD. Differences between the two groups of placentas were analysed by the Student's t-test. The differences were considered statistically significant at p-value less than 0.05.

Results

The presence of IGF and insulin receptors in placental preparations was detected by immunoblotting after lectin-affinity chromatography. Eluates from all lectin columns were analysed, but receptors were detected only in WGA, RCA I, SNA, LCA, Con A, PHA-E and PHA-L eluates. The same pattern of immunoreactive bands on immunoblots was seen for both diabetic and healthy placentas. In *Figure 1* rep-

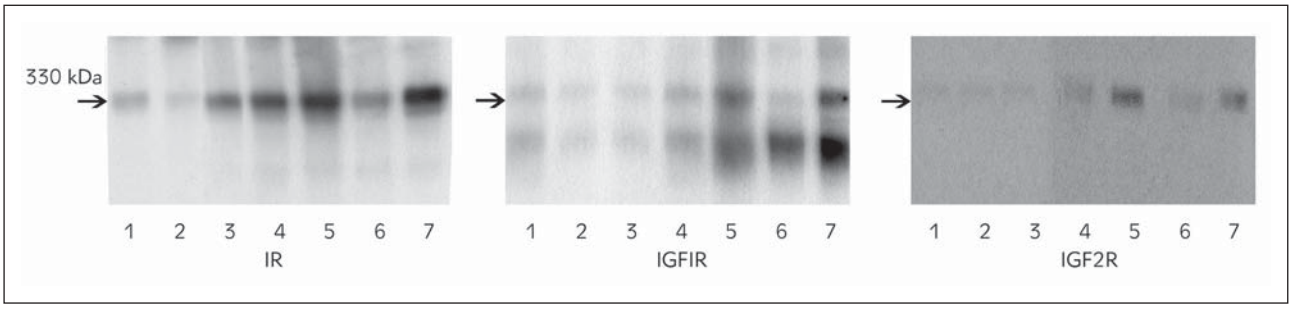

Figure 1 Detection of IGF and insulin receptors from diabetic placentas bound to the immobilised lectins. Lectin-bound fractions obtained after affinity chromatography were dialysed, concentrated and subjected to non-reducing SDS-PAGE followed by immunoblotting for IR, IGF1R and IGF2R. Samples were eluted from agarose-bound PHA-E (lane 1), PHA-L (lane 2), Con A (lane 3), LCA (lane 4), SNA (lane 5), WGA (lane 6) and RCA I (lane 7).

Table II Lectin-bound radioactivity of ^{125}I-insulin, ^{125}I-des(1-3)IGF-I and ^{125}I-IGF-II expressed as a percentage of the total radioactivity (mean ± SD, n = 5 for each group) using receptors' preparations from diabetic placentas (DP) and healthy placentas (HP).

Ligand	^{125}I-Insulin (%)		^{125}I-des(1-3)-IGF-I (%)		^{125}I-IGF-II (%)	
	DP	HP	DP	HP	DP	HP
WGA	3.5±0.84	2.7±1.15	8.7±2.08	9.5±0.71	17.3±1.53	18.5±3.54
s-WGA	1.3±0.58	1.5±0.71	1.3±0.58	2.5±0.71	4.7±0.58	4.5±2.12
RCA I	1.7±0.58	2.0±0.00	13.0±1.73	16.5±0.71	12.0±1.10	10.5±2.12
SNA	1.2±0.41	1.3±0.58	6.3±1.53	8.5±0.71	21.7±1.53	26.5±0.61
ECL	1.7±0.58	1.5±0.71	11.3±1.15	15.5±0.71	8.7±0.58	7.67±3.06
UEA I	1.3±0.58	1.5±0.71	2.3±0.58	3.7±0.58	3.0±0.10	2.5±0.71
LCA	3.3±1.53	2.5±0.71	7.1±0.96	8.2±0.63	8.5±1.06	7.9±1.06
Con A	1.8±1.17	2.3±1.53	8.7±1.53	12.5±0.71	10.3±1.15	11.5±0.71
PHA-E	1.7±0.96	2.5±0.71	13.7±2.08	13.7±0.58	14.7±1.15	14.5±0.71
PHA-L	1.7±1.15	2.5±0.71	7.3±0.58	8.7±0.58	8.3±0.58	9.5±0.71
MAA1	1.3±0.58	1.3±0.58	1.3±0.58	1.3±0.58	1.3±0.58	1.3±0.58

resentative immunoblots obtained with receptors isolated from diabetic placentas are shown.

Reactivity of the IGF and insulin receptors towards eleven immobilised lectins was also monitored by measuring radioactivity within ^{125}I-ligand/receptor complexes after their binding to immobilised lectins. The preparations of receptors originating from the placentas of mothers diagnosed with pregestational IDDM were compared to those of healthy mothers. The values of lectin-bound radioactivity are given as percentage of total radioactivity in *Table II*.

^{125}I-insulin/receptor complexes were the most reactive towards WGA, RCA I, ECL, LCA, Con A, PHA-E and PHA-L. In general, their reactivity was much lower than the reactivity of ^{125}I-des(1-3)IGF-I/receptor and ^{125}I-IGF-II/receptor complexes towards the same lectins. The last two ^{125}I-ligand/receptor complexes bound significantly to SNA as well.

Statistical analysis demonstrated that there was no difference in ^{125}I-ligand/receptor binding to lectins between the samples from mothers with IDDM and healthy mothers.

Discussion

In this study we demonstrated that the content and type of N-glycans covalently bound to IGF and insulin receptors in placentas from mothers diag-

nosed with pregestational IDDM (and therapeutically treated) do not differ from the receptors in placentas from healthy mothers.

Different physiological and pathological conditions have long been known to be associated with alteration in glycosylation (11–13), which may affect the biochemical and functional characteristics of glycoproteins. The glycosylation of proteins in cancer is the most attractive area of research, as it frequently leads to the discovery of new glycosylation patterns of the examined proteins (11, 14). Altered glycosylation affects the localisation of glycoproteins, their half-life and biological activity (11). Diabetes mellitus is a complex disease, with altered glycemia and insulinemia. Although several lines of evidence suggest that insulin resistance and diabetes mellitus may lead to changes in the glycome, very little is known about this (15, 16). For example, it was shown that alterations in the activity of the enzymes involved in glycosylation result in changes in the sialic acid (Sia) and Fuc content in insulin-resistant patients and animals (17–19). Insulin deficiency can lead to an increase in the plasma concentration of Sia (19). Parry and colleagues investigated the effect of chronic insulin resistance on the glycosylation of rat muscle and liver proteins. Using the mass spectrometric analysis of the glycans from these cells they obtained very similar profiles, suggesting that any changes in glycosylation most likely occur on specific proteins, rather than globally (20). In analysing the saccharide content of glycoconjugates in the human placental cells from pregnancies complicated by altered glycemia (minor degree of glucose intolerance or gestational diabetes mellitus), Sgambati et al. (21) showed a decrease in the content of Sia α2,6-linked to galactose (Gal)/GlcNAc and increase in GlcNAc in the placentas from pathological groups, in particular in gestational diabetes mellitus. On the other hand, the placentas from mothers with a minor degree of glucose intolerance had an increase of UEA I-reactive L-Fuc (21). Women with pregestational IDDM were not examined.

Here we tested whether the N-glycans attached to IGF and insulin receptors from the term placentas of mothers with pregestational IDDM are different from those obtained from the term placentas of healthy mothers. We, therefore, analysed the type of N-glycans and content of terminal saccharide residues covalently bound to IGF and insulin receptors by lectin-affinity chromatography. The proper choice of lectins, whose saccharide specificities have been elucidated in detail and which cover the reactivity towards the great range of saccharides that can be found on (mostly N-) glycans, enabled such investigation. The results obtained indicate that IGF and insulin receptors in the placenta of mothers with IDDM mostly possess biantennary complex type N-glycans (Con A), although branched glycans are also present (PHA-L). The core Fuc (LCA) and bisecting GlcNAc (RCA I, PHA-E) residues are frequently present on these glycan structures. The oligosaccharides terminate with terminal Fuc (UEA I) and α2,6-Sia (SNA), but not with α2,3-Sia (MAA).

In this study we did not find any differences in the content and type of N-glycans covalently bound to IGF and insulin receptors between the placentas obtained from mothers diagnosed with pregestational IDDM and the placentas obtained from healthy mothers. Since the mothers with pregestational IDDM were on adequate insulin therapy (which prevented the development of vascular and renal disease or increase in the content of HbA1c), it might be that the tight glycemic control prevented alterations in the glycosylation of IGF and insulin receptors, which are crucial for fetal growth and development.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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